IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

AJINOMOTO HEARTLAND LLC, a Delaware Corporation, Plaintiff, v. **GLOBAL BIO-CHEM TEHCNOLOGY GROUP COMPANY, LIMITED,** a Cayman Islands Corporation; **CHANGCHUN DACHENG BIO-CHEM ENGINEERING DEVELOPMENT COMPANY, LIMITED,** a People's Republic of China Corporation; **CHANGCHUN BAOCHENG BIO-CHEM DEVELOPMENT COMPANY, LIMITED,** a People's Republic of China Corporation; **CHANGCHUN DAHE BIO TECHNOLOGY DEVELOPMENT COMPANY, LIMITED,** a People's Republic of China Corporation; **BIO-CHEM TECHNOLOGY (HK) LIMITED,** a Hong Kong Special Administrative Region Corporation, Defendants.	C. A. No. _____

COMPLAINT

Plaintiff, Ajinomoto Heartland LLC, for its complaint against defendants, Global Bio-Chem Technology Group Company Limited, Changchun Dacheng Bio-Chem Engineering Development Company, Limited, Changchun Baocheng Bio-Chem Development Company, Limited, Changchun Dahe Bio Technology Development Company, Limited, Bio-Chem Technology (HK) Limited, alleges as follows:

Subject Matter Jurisdiction

1. This is an action for patent infringement arising from Title 35 of the United States Code. 35 U.S.C §§ 1 *et seq.*, arising from the making, using, offering for sale, sale, and importation of L-lysine products. Subject matter jurisdiction over this matter is conferred upon the Court pursuant to 28 U.S.C. §§ 1331 and 1338(a).

The Parties

2. Plaintiff Ajinomoto Heartland LLC ("Heartland") is a corporation organized under the laws of the State of Delaware having its principal place of business at 8430 W. Bryn Mawr Avenue, Suite 650, Chicago, Illinois, 60631.

3. On information and belief Defendant Global Bio-Chem Technology Group Company Limited ("GBT") is a corporation organized under the laws of the Cayman Islands having its principal place of business at Unit 1104, Admiralty Centre, Tower 1, 18 Harcourt Road, Admiralty, Hong Kong. GBT is a publicly traded corporation.

4. On information and belief Defendant Changchun Dacheng Bio-Chem Engineering Development Company, Limited ("Dacheng") is a corporation organized under the laws of the People's Republic of China having its principal place of business at No. 886 Xihuangcheng Road, Processing Corn District, Changchun Economic and Technological Development Zone, Jilin Province, China. GBT owns a majority interest in Dacheng.

5. On information and belief Defendant Changchun Baocheng Bio-Chem Development Company, Limited ("Baocheng") is a corporation organized under the laws

of the People's Republic of China having its principal place of business at No. 886 Xihuangcheng Road, Processing Corn District, Changchun Economic and Technological Development Zone, Jilin Province, China. GBT owns a majority interest in Baocheng.

6. On information and belief Defendant Changchun Dahe Bio Technology Development Company, Limited ("Dahe Bio") is a corporation organized under the laws of the People's Republic of China having its principal place of business at No. 28 Xihuangcheng Road, Processing Corn District, Changchun Economic and Technological Development Zone, Jilin Province, China. GBT owns a one hundred percent (100%) equity interest in Dahe Bio.

7. On information and belief Defendant Bio-Chem Technology (HK) Limited ("BCT") is a corporation organized under the laws of the Hong Kong Special Administrative Region of the People's Republic of China having its principal place of business at Unit 1104, Admiralty Centre, Tower 1, 18 Harcourt Road, Admiralty, Hong Kong. GBT owns a one hundred percent (100%) equity interest in BCT.

Venue and Personal Jurisdiction

4. Venue is proper in this forum pursuant to 28 U.S.C. § 1391(d), 28 U.S.C. § 1400(b) and the applicable law of this Court. Venue and jurisdiction for this action are proper in this Court given that the Defendants are alien corporations.

The Patents-in-Suit

5. Heartland is the exclusive licensee of United States Patent No. 5,827,698 ("the '698 patent") to Yoshimi Kikuchi, Tomoko Suzuki and Hiroyuki Kojima, entitled LYSINE DECARBOXYLASE GENE AND METHOD OF PRODUCING L-LYSINE

issued October 27, 1998. Pursuant to local rule 3.2, a copy of the '698 patent is appended hereto as Exhibit A.

6. Heartland is the exclusive licensee of United States Patent No. 6,040,160 ("the '160 patent") to Hiroyuki Kojima, Yuri Ogawa, Kazue Kawamura and Konosuke Sano, entitled METHOD OF PRODUCING L-LYSINE BY FERMENTATION issued March 21, 2000. Pursuant to local rule 3.2, a copy of the '160 patent is appended hereto as Exhibit B.

Allegations of Direct Infringement
(35 U.S.C. § 271(g))

8. On information and belief, GBT's making, using, offering to sell, selling and importing L-lysine Mono HCl Feed Grade (minimum lysine equivalent content of 98%) and Protein L-lysine/L-Lysine Sulphate Feed Grade (minimum lysine equivalent content of 65%) ("GBT's L-lysine products"), constitute willful infringement of one or more claims of the '698 patent in violation of 35 U.S.C. § 271(g).

9. On information and belief, GBT's making, using, offering to sell, selling and importing GBT's L-lysine products, constitute willful infringement of one or more claims of the '160 patent in violation of 35 U.S.C. § 271(g).

10. On information and belief, GBT's willful infringement will continue unless it is enjoined therefrom.

11. On information and belief, Dacheng's making, using, offering to sell, selling and importing GBT's L-lysine products, constitute willful infringement of one or more claims of the '698 patent in violation of 35 U.S.C. § 271(g).

12. On information and belief, Dacheng's making, using, offering to sell, selling and importing GBT's L-lysine products, constitute willful infringement of one or more claims of the '160 patent in violation of 35 U.S.C. § 271(g).

13. On information and belief, Dacheng's willful infringement will continue unless it is enjoined therefrom.

14. On information and belief, Baocheng's making, using, offering to sell, selling and importing GBT's L-lysine products, constitute willful infringement of one or more claims of the '698 patent in violation of 35 U.S.C. § 271(g).

15. On information and belief, Baocheng's making, using, offering to sell, selling and importing GBT's L-lysine products, constitute willful infringement of one or more claims of the '160 patent in violation of 35 U.S.C. § 271(g).

16. On information and belief, Baocheng's willful infringement will continue unless it is enjoined therefrom.

17. On information and belief, Dahe Bio's making, using, offering to sell, selling and importing GBT's L-lysine products, constitute willful infringement of one or more claims of the '698 patent in violation of 35 U.S.C. § 271(g).

18. On information and belief, Dahe Bio's making, using, offering to sell, selling and importing GBT's L-lysine products, constitute willful infringement of one or more claims of the '160 patent in violation of 35 U.S.C. § 271(g).

19. On information and belief, Dahe Bio's willful infringement will continue unless it is enjoined therefrom.

20. On information and belief, BCT's making, using, offering to sell, selling and importing GBT's L-lysine products, constitute willful infringement of one or more claims of the '698 patent in violation of 35 U.S.C. § 271(g).

21. On information and belief, BCT's making, using, offering to sell, selling and importing GBT's L-lysine products, constitute willful infringement of one or more claims of the '160 patent in violation of 35 U.S.C. § 271(g).

22. On information and belief, BCT's willful infringement will continue unless it is enjoined therefrom.

Request for Relief

Plaintiff, Heartland respectfully requests:

a. Judgment that GBT, Dacheng, Baocheng, Dahe Bio and BCT infringe one or more claims of the '698 and '160 patents (35 U.S.C. § 271(g));

b. Judgment that GBT's, Dacheng's, Baocheng's, Dahe Bio's and BCT's infringement of the claims of the '698 and '160 patents is willful;

c. A preliminary and permanent injunction against continued infringement (35 U.S.C. § 283);

d. An award of damages to redress GBT's, Dacheng's, Baocheng's, Dahe Bio's and BCT's infringement (35 U.S.C. § 284);

e. Increased and trebled damages for GBT's, Dacheng's, Baocheng's, Dahe Bio's and BCT's willful infringement (35 U.S.C. § 284);

f. Heartland's attorney fees (35 U.S.C. § 285);

g. Heartland's costs (Rule 54(d), Fed. R. Civ. P.).

OF COUNSEL:

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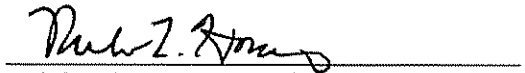
Dated: April 25, 2006

729105/30162

POTTER ANDERSON & CORROON LLP

By: 

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JS 44 (Rev 3/99)

CIVIL COVER SHEET

The JS-44 civil cover sheet and the information contained herein neither replace nor supplement the filing and service of pleadings or other papers as required by law, except as provided by local rules of court. This form, approved by the Judicial Conference of the United States in September 1974, is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet. (SEE INSTRUCTIONS ON THE REVERSE OF THE FORM.)

I. (a) PLAINTIFFS

AJINOMOTO HEARTLAND LLC

(b) County of Residence of First Listed Plaintiff _____
(EXCEPT IN U.S. PLAINTIFF CASES)

DEFENDANTS

SEE LIST OF DEFENDANTS, attached hereto as EXHIBIT A

County of Residence of First Listed Defendant _____
(IN U.S. PLAINTIFF CASES ONLY)
NOTE: IN LAND CONDEMNATION CASES, USE THE LOCATION OF THE LAND INVOLVED

(c) Attorney's (Firm Name, Address, and Telephone Number)

Potter Anderson & Corroon LLP
Hercules Plaza, 6th Floor
1313 N. Market Street, P.O. Box 951
Wilmington, DE 19899-0951

Attorneys (If Known)

II. BASIS OF JURISDICTION (Place an "X" in One Box Only)

- [ ] 1 U.S. Government Plaintiff
- [x] 3 Federal Question (U.S. Government Not a Party)
- [ ] 2 U.S. Government Defendant
- [ ] 4 Diversity (Indicate Citizenship of Parties in Item III)

III. CITIZENSHIP OF PRINCIPAL PARTIES (Place an "X" in One Box for Plaintiff and One Box for Defendant)
(For Diversity Cases Only)

	PTF	DEF		PTF	DEF
Citizen of This State	[ ] 1	[ ] 1	Incorporated *or* Principal Place of Business In This State	[ ] 4	[ ] 4
Citizen of Another State	[ ] 2	[ ] 2	Incorporated *and* Principal Place of Business In Another State	[ ] 5	[ ] 5
Citizen or Subject of a Foreign Country	[ ] 3	[ ] 3	Foreign Nation	[ ] 6	[ ] 6

IV. NATURE OF SUIT (Place an "X" in One Box Only)

CONTRACT
- [ ] 110 Insurance
- [ ] 120 Marine
- [ ] 130 Miller Act
- [ ] 140 Negotiable Instrument
- [ ] 150 Recovery of Overpayment & Enforcement of Judgment
- [ ] 151 Medicare Act
- [ ] 152 Recovery of Defaulted Student Loans (Excl. Veterans)
- [ ] 153 Recovery of Overpayment of Veteran's Benefits
- [ ] 160 Stockholders' Suits
- [ ] 190 Other Contract
- [ ] 195 Contract Product Liability

REAL PROPERTY
- [ ] 210 Land Condemnation
- [ ] 220 Foreclosure
- [ ] 230 Rent Lease & Ejectment
- [ ] 240 Torts to Land
- [ ] 245 Tort Product Liability
- [ ] 290 All Other Real Property

TORTS

PERSONAL INJURY
- [ ] 310 Airplane
- [ ] 315 Airplane Product Liability
- [ ] 320 Assault, Libel & Slander
- [ ] 330 Federal Employers' Liability
- [ ] 340 Marine
- [ ] 345 Marine Product Liability
- [ ] 350 Motor Vehicle
- [ ] 355 Motor Vehicle Product Liability
- [ ] 360 Other Personal Injury

PERSONAL INJURY
- [ ] 362 Personal Injury—Med. Malpractice
- [ ] 365 Personal Injury—Product Liability
- [ ] 368 Asbestos Personal Injury Product Liability

PERSONAL PROPERTY
- [ ] 370 Other Fraud
- [ ] 371 Truth in Lending
- [ ] 380 Other Personal Property Damage
- [ ] 385 Property Damage Product Liability

CIVIL RIGHTS
- [ ] 441 Voting
- [ ] 442 Employment
- [ ] 443 Housing/ Accommodations
- [ ] 444 Welfare
- [ ] 440 Other Civil Rights

PRISONER PETITIONS
- [ ] 510 Motions to Vacate Sentence
- Habeas Corpus:
- [ ] 530 General
- [ ] 535 Death Penalty
- [ ] 540 Mandamus & Other
- [ ] 550 Civil Rights
- [ ] 555 Prison Condition

FORFEITURE/PENALTY
- [ ] 610 Agriculture
- [ ] 620 Other Food & Drug
- [ ] 625 Drug Related Seizure of Property 21 USC
- [ ] 630 Liquor Laws
- [ ] 640 R.R. & Truck
- [ ] 650 Airline Regs.
- [ ] 660 Occupational Safety/Health
- [ ] 690 Other

LABOR
- [ ] 710 Fair Labor Standards Act
- [ ] 720 Labor/Mgmt. Relations
- [ ] 730 Labor/Mgmt. Reporting & Disclosure Act
- [ ] 740 Railway Labor Act
- [ ] 790 Other Labor Litigation
- [ ] 791 Empl. Ret. Inc. Security Act

BANKRUPTCY
- [ ] 422 Appeal 28 USC 158
- [ ] 423 Withdrawal 28 USC 157

PROPERTY RIGHTS
- [ ] 820 Copyrights
- [x] 830 Patent
- [ ] 840 Trademark

SOCIAL SECURITY
- [ ] 861 HIA (1395ff)
- [ ] 862 Black Lung (923)
- [ ] 863 DIWC/DIWW (405(g))
- [ ] 864 SSID Title XVI
- [ ] 865 RSI (405(g))

FEDERAL TAX SUITS
- [ ] 870 Taxes (U.S. Plaintiff or Defendant)
- [ ] 871 IRS—Third Party 26 USC 7609

OTHER STATUTES
- [ ] 400 State Reapportionment
- [ ] 410 Antitrust
- [ ] 430 Banks and Banking
- [ ] 450 Commerce/ICC Rates/etc
- [ ] 460 Deportation
- [ ] 470 Racketeer Influenced and Corrupt Organizations
- [ ] 810 Selective Service
- [ ] 850 Securities/Commodities/ Exchange
- [ ] 875 Customer Challenge 12 USC 3410
- [ ] 891 Agricultural Acts
- [ ] 892 Economic Stabilization Act
- [ ] 893 Environmental Matters
- [ ] 894 Energy Allocation Act
- [ ] 895 Freedom of Information Act
- [ ] 900 Appeal of Fee Determination Under Equal Access to Justice
- [ ] 950 Constitutionality of State Statutes
- [ ] 890 Other Statutory Actions

V. ORIGIN (PLACE AN "X" IN ONE BOX ONLY)

- [x] 1 Original Proceeding
- [ ] 2 Removed from State Court
- [ ] 3 Remanded from Appellate Court
- [ ] 4 Reinstated or Reopened
- [ ] 5 Transferred from another district (specify)
- [ ] 6 Multidistrict Litigation
- [ ] 7 Appeal to District Judge from Magistrate Judgment

VI. CAUSE OF ACTION (Cite the U.S. Civil Statute under which you are filing and write brief statement of cause. Do not cite jurisdictional statutes unless diversity.)

35 U.S.C. Sections 1, et seq., including 35 U.S.C. Section 271(g)

VII. REQUESTED IN COMPLAINT: [ ] CHECK IF THIS IS A CLASS ACTION UNDER F.R.C.P. 23 DEMAND $ _____ CHECK YES only if demanded in complaint: JURY DEMAND: [ ] Yes [x] No

VIII. RELATED CASE(S) IF ANY (See instructions): JUDGE _____ DOCKET NUMBER _____

DATE
04/25/2006

SIGNATURE OF ATTORNEY OF RECORD

FOR OFFICE USE ONLY

RECEIPT # _____ AMOUNT _____ APPLYING IFP _____ JUDGE _____ MAG. JUDGE _____

JS 44 Reverse (Rev 12/96)

INSTRUCTIONS FOR ATTORNEYS COMPLETING CIVIL COVER SHEET FORM JS-44

Authority For Civil Cover Sheet

The JS-44 civil cover sheet and the information contained herein neither replaces nor supplements the filings and service of pleading or other papers as required by law, except as provided by local rules of court This form, approved by the Judicial Conference of the United States in September 1974, is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet Consequently, a civil cover sheet is submitted to the Clerk of Court for each civil complaint filed The attorney filing a case should complete the form as follows:

I. **(a) Plaintiffs-Defendants.** Enter names (last, first, middle initial) of plaintiff and defendant If the plaintiff or defendant is a government agency, use only the full name or standard abbreviations If the plaintiff or defendant is an official within a government agency, identify first the agency and then the official, giving both name and title

(b) County of Residence For each civil case filed, except U S plaintiff cases, enter the name of the county where the first listed plaintiff resides at the time of filing In U S plaintiff cases, enter the name of the county in which the first listed defendant resides at the time of filing (NOTE: In land condemnation cases, the county of residence of the "defendant" is the location of the tract of land involved)

(c) Attorneys Enter the firm name, address, telephone number, and attorney of record If there are several attorneys, list them on an attachment, noting in this section "(see attachment)"

II. **Jurisdiction** The basis of jurisdiction is set forth under Rule 8(a), F R C P , which requires that jurisdictions be shown in pleadings Place an "X" in one of the boxes If there is more than one basis of jurisdiction, precedence is given in the order shown below

United States plaintiff (1) Jurisdiction based on 28 U S C 1345 and 1348 Suits by agencies and officers of the United States, are included here

United States defendant (2) When the plaintiff is suing the United States, its officers or agencies, place an "X" in this box

Federal question (3) This refers to suits under 28 U S C 1331, where jurisdiction arises under the Constitution of the United States, an amendment to the Constitution, an act of Congress or a treaty of the United States In cases where the U S is a party, the U S plaintiff or defendant code takes precedence, and box 1 or 2 should be marked

Diversity of citizenship (4) This refers to suits under 28 U S C 1332, where parties are citizens of different states When Box 4 is checked, the citizenship of the different parties must be checked (See Section III below; federal question actions take precedence over diversity cases)

III. **Residence (citizenship) of Principal Parties.** This section of the JS-44 is to be completed if diversity of citizenship was indicated above Mark this section for each principal party

IV. **Nature of Suit** Place an "X" in the appropriate box If the nature of suit cannot be determined, be sure the cause of action, in Section IV below, is sufficient to enable the deputy clerk or the statistical clerks in the Administrative Office to determine the nature of suit If the cause fits more than one nature of suit, select the most definitive

V. **Origin** Place an "X" in one of the seven boxes

Original Proceedings (1) Cases which originate in the United States district courts

Removed from State Court (2) Proceedings initiated in state courts may be removed to the district courts under Title 28 U S C , Section 1441 When the petition for removal is granted, check this box

Remanded from Appellate Court (3) Check this box for cases remanded to the district court for further action Use the date of remand as the filing date

Reinstated or Reopened (4) Check this box for cases reinstated or reopened in the district court Use the reopening date as the filing date

Transferred from Another District (5) For cases transferred under Title 28 U S C Section 1404(a) Do not use this for within district transfers or multidistrict litigation transfers

Multidistrict Litigation (6) Check this box when a multidistrict case is transferred into the district under authority of Title 28 U S C Section 1407 When this box is checked, do not check (5) above

Appeal to District Judge from Magistrate Judgment (7) Check this box for an appeal from a magistrate judge's decision

VI. **Cause of Action** Report the civil statute directly related to the cause of action and give a brief description of the cause

VII. **Requested in Complaint** Class Action Place an "X" in this box if you are filing a class action under Rule 23, F R Cv P

Demand In this space enter the dollar amount (in thousands of dollars) being demanded or indicate other demand such as a preliminary injunction

Jury Demand Check the appropriate box to indicate whether or not a jury is being demanded

VIII. **Related Cases** This section of the JS-44 is used to reference related pending cases if any If there are related pending cases, insert the docket numbers and the corresponding judge names for such cases

Date and Attorney Signature Date and sign the civil cover sheet

ATTACHMENT TO CIVIL COVER SHEET

IN THE UNITED STATES DISTRICT COURT
FOR DISTRICT OF DELAWARE

AJINOMOTO HEARTLAND LLC,
a Delaware Corporation,

Plaintiff,

v. C. A. No. _____

GLOBAL BIO-CHEM TEHCNOLOGY GROUP COMPANY LIMITED,
a Cayman Islands Corporation;

CHANGCHUN DACHENG BIO-CHEM ENGINEERING DEVELOPMENT COMPANY, LIMITED,
a People's Republic of China Corporation;

CHANGCHUN BAOCHENG BIO-CHEM DEVELOPMENT COMPANY, LIMITED,
a People's Republic of China Corporation;

CHANGCHUN DAHE BIO TECHNOLOGY DEVELOPMENT COMPANY, LIMITED,
a People's Republic of China Corporation;

BIO-CHEM TECHNOLOGY (HK) LIMITED,
a Hong Kong Special Administrative Region Corporation,

Defendants.

729143/30162

EXHIBIT A

US005827698A

United States Patent [19]

Kikuchi et al.

[11] Patent Number: 5,827,698

[45] Date of Patent: Oct. 27, 1998

[54] **LYSINE DECARBOXYLASE GENE AND METHOD OF PRODUCING L-LYSINE**

[75] Inventors: **Yoshimi Kikuchi; Tomoko Suzuki; Hiroyuki Kojima**, all of Kawasaki, Japan

[73] Assignee: **Ajinomoto Co., Inc.**, Tokyo, Japan

[21] Appl. No.: **849,212**

[22] PCT Filed: **Dec. 5, 1995**

[86] PCT No.: **PCT/JP95/02481**

§ 371 Date: **Jun. 9, 1997**

§ 102(e) Date: **Jun. 9, 1997**

[87] PCT Pub No.: **WO96/17930**

PCT Pub Date: **Jun. 13, 1996**

[30] **Foreign Application Priority Data**

Dec. 9, 1994 [JP] Japan 6-306386

[51] **Int. Cl.**[6] **C12P 13/08**; C07H 21/00; C07H 21/02; C07H 21/04

[52] **U.S. Cl.** **435/115**; 530/350; 435/71.1; 435/29; 435/183; 435/252.8; 536/23.1; 536/23.2

[58] **Field of Search** 530/350; 435/71.1, 435/29, 183, 252.8, 115; 536/23.2, 23.1

[56] **References Cited**

PUBLICATIONS

CA 111:209849 1989.

CA 1056:36506 1986.

Meng et al., J. Bacteriology vol. 174 p. 2659. 1992.

Primary Examiner—Sheela Huff

Attorney, Agent, or Firm—Oblon, Spivak, McClelland, Maier & Neustadt, P.C.

[57] **ABSTRACT**

L-lysine is produced efficiently by cultivating, in a liquid medium, a microorganism belonging to the genus Escherichia with decreased or disappeared lysine decarboxylase activity relevant to decomposition of L-lysine, for example, a bacterium belonging to the genus Escherichia with restrained expression of a novel gene coding for lysine decarboxylase and/or a known gene cadA to allow L-lysine to be produced and accumulated in a culture liquid, and collecting it.

22 Claims, 3 Drawing Sheets

FIG. 1

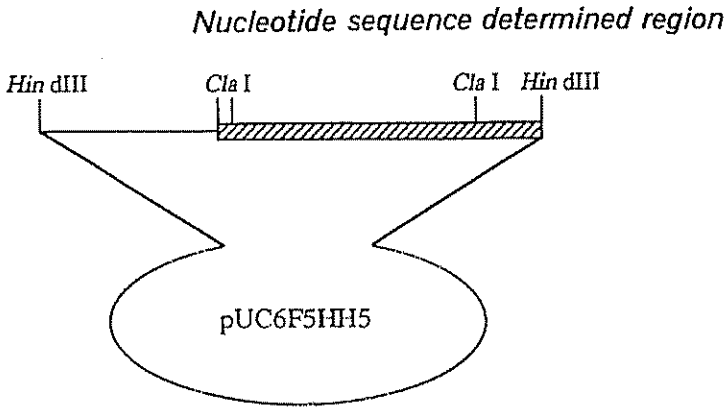

Nucleotide sequence determined region
Hin dIII
Cla I
Cla I
Hin dIII
pUC6F5HH5

FIG. 2

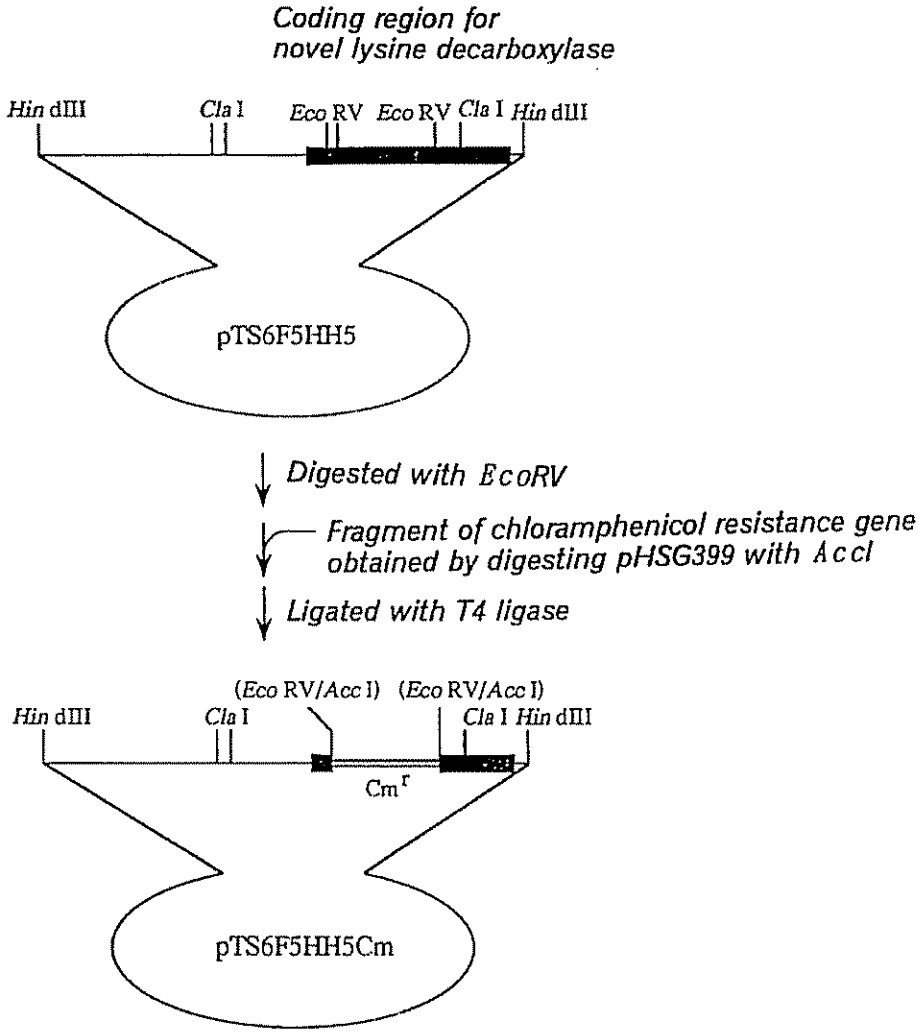
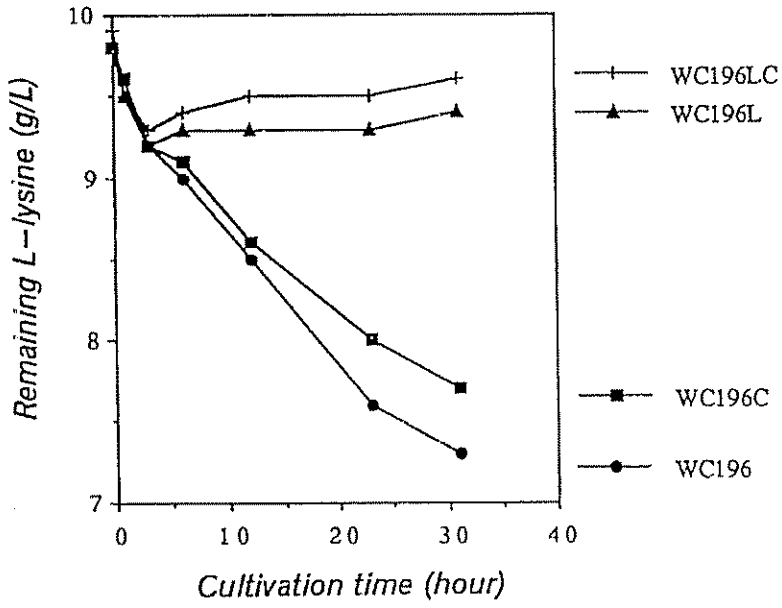

Coding region for
novel lysine decarboxylase
Hin dIII
Cla I
Eco RV
Eco RV
Cla I
Hin dIII
pTS6F5HH5
Digested with EcoRV
Fragment of chloramphenicol resistance gene
obtained by digesting pHSG399 with AccI
Ligated with T4 ligase
(Eco RV/Acc I)
(Eco RV/Acc I)
Hin dIII
Cla I
Cla I
Hin dIII
Cm r
pTS6F5HH5Cm

FIG. 3


10
9
8
7
0
10
20
30
40
Remaining L-lysine (g/L)
Cultivation time (hour)
WC196LC
WC196L
WC196C
WC196

LYSINE DECARBOXYLASE GENE AND METHOD OF PRODUCING L-LYSINE

TECHNICAL FIELD

The present invention relates to a novel lysine decarboxylase gene of *Escherichia coli* relevant to decomposition of L-lysine, a microorganism belonging to the genus Escherichia with restrained expression of the gene and/or another lysine decarboxylase gene known as cadA gene, and a method of producing L-lysine by using the microorganism Recently, the demand of L-lysine as a feed additive actively increases.

BACKGROUND ART

Lysine decarboxylase, which catalyzes a reaction to produce cadaverine by decarboxylation of L-lysine, is known as an L-lysine-decomposing enzyme of *Escherichia coli*. A nucleotide sequence of its gene called cadA, and an amino acid sequence encoded by the gene have been already reported (Meng, S. and Bennett, G. N., *J. Bacteriol.*, 174, 2659 (1992)). There are two reports for lysine decarboxylase encoded by a gene other than cadA of *Escherichia coli*, which describe that faint activity was detected in a mutant strain of *Escherichia coli* (Goldemberg, S. H., *J. Bacteriol.*, 141, 1428 (1980); Wertheimer, S. J. and Leifer, Z., *Biochem. Biophys. Res. Commun.*, 114, 882 (1983)). However, it was reported for this activity by Goldemberg, S. H. that the enzyme activity decreased in a degree of about 30% after a heat treatment at 60° C. for 4 minutes, while it was reported by Wertheimer, S. J. et al. that no such phenomenon was observed. Accordingly, the presence of the second lysine decarboxylase is indefinite.

On the other hand, L-lysine is produced by known methods for using *Escherichia coli*, including a method comprising cultivating a mutant strain resistant to lysine analog or a recombinant strain harboring a vector with incorporated deoxyribonucleic acid which carries genetic information relevant to L-lysine biosynthesis (Japanese Patent Laid-open No. 56-18596). However, there is no report at all for L-lysine production by using a microorganism belonging to the genus Escherichia with restrained expression of the lysine decarboxylase gene.

DISCLOSURE OF THE INVENTION

An object of the present invention is to obtain a novel lysine decarboxylase gene of *Escherichia coli*, create an L-lysine-producing microorganism belonging to the genus Escherichia with restrained expression of the gene and/or the cadA gene, and provide a method of producing L-lysine by cultivating the microorganism belonging to the genus Escherichia. When the present inventors created an *Escherichia coli* strain in which the cadA gene as a known lysine decarboxylase gene was destroyed, it was found that cadaverine as a decomposition product of L-lysine by lysine decarboxylase was still produced in this microbial strain. Thus the present inventors assumed that a novel lysine decarboxylase gene should be present in *Escherichia coli*, and it might greatly affect fermentative production of L-lysine by using a microorganism belonging to the genus Escherichia. As a result of trials to achieve cloning of the gene, the present inventors succeeded in obtaining a novel lysine decarboxylase gene different from the cadA gene. It was also found that the L-lysine-decomposing activity was remarkably decreased or disappeared, and the L-lysine productivity was significantly improved by restraining expression of this gene, and restraining expression of the cadA gene in an L-lysine-producing microorganism of *Escherichia coli*. Thus the present invention was completed.

Namely, the present invention provides a novel gene which codes for lysine decarboxylase originating from *Escherichia coli*. This gene has been designated as "ldc" gene.

In another aspect, the present invention provides a microorganism belonging to the genus Escherichia having L-lysine productivity with decreased or disappeared lysine decarboxylase activity in cells.

In still another aspect, the present invention provides a method of producing L-lysine comprising the steps of cultivating, in a liquid medium, the microorganism belonging to the genus Escherichia described above to allow L-lysine to be produced and accumulated in a culture liquid, and collecting it.

The microorganism belonging to the genus Escherichia described above includes a microorganism in which lysine decarboxylase activity in cells is decreased or disappeared by restraining expression of the ldc gene and/or the cadA gene.

The present invention will be described in detail below.

<1>Preparation of DNA fragment containing novel lysine decarboxylase gene

A DNA fragment containing the novel lysine decarboxylase gene (ldc) of the present invention can be obtained as follows from an available strain of *Escherichia coli*, for example, K-12 strain or a derivative strain therefrom.

At first, the cadA gene, which is a gene of known lysine decarboxylase, is obtained from chromosomal DNA of W3110 strain originating from *Escherichia coli* K-12 by using a polymerase chain reaction method (hereinafter referred to as "PCR method"). The nucleotide sequence of the cadA gene, and the amino acid sequence encoded by it are shown in SEQ ID NOS:5 and 6 respectively. DNA fragments having sequences similar to the cadA gene are cloned from a chromosomal DNA library of *Escherichia coli* W3110 in accordance with a method for using a plasmid vector or a phage vector to confirm whether or not the novel lysine decarboxylase gene is contained in the DNA fragments. The confirmation of the fact that the objective gene is contained can be performed in accordance with a Southern hybridization method by using a probe prepared by the PCR method.

A nucleotide sequence of the gene contained in the DNA fragment thus obtained is determined as follows. At first, the DNA fragment is ligated with a plasmid vector autonomously replicable in cells of *Escherichia coli* to prepare recombinant DNA which is introduced into competent cells of *Escherichia coli*. An obtained transformant is cultivated in a liquid medium, and the recombinant DNA is recovered from proliferated cells. An entire nucleotide sequence of the DNA fragment contained in the recovered recombinant DNA is determined in accordance with a dideoxy method (Sanger, F. et al., *Proc. Natl. Acad. Sci.*, 74, 5463 (1977)). The structure of DNA is analyzed to determine existing positions of promoter, operator, SD sequence, initiation codon, termination codon, open reading frame, and so on.

The novel lysine decarboxylase gene of the present invention has a sequence from 1005–1007th ATG to 3141–3143rd GGA of the entire nucleotide sequence of the DNA fragment shown in SEQ ID NO:3 in Sequence Listing. This gene codes for lysine decarboxylase having an amino acid sequence shown in SEQ ID NO:4 in Sequence Listing. It has been found that the homology between the novel lysine

decaroboxylase and the lysine decaroboxylase coded by cadA gene is 69.4%.

The gene of the present invention may be those which code for lysine decarboxylase having the amino acid sequence shown in SEQ ID NO:4 in Sequence Listing, a nucleotide sequence of which is not limited to the nucleotide sequence described above. The lysine decarboxylase encoded by the gene of the present invention may have substitution, deletion, or insertion of one or a plurality of amino acid residues without substantial deterioration of the lysine decarboxylase activity, in the amino acid sequence described above. Genes which code for lysine decarboxylase having such deletion, insertion, or substitution can be obtained from variants, spontaneous mutant strains, or artificial mutant strains of *Escherichia coli*, or from microorganisms belonging to the genus Escherichia other than *Escherichia coli*. The mutant genes which code for lysine decarboxylase having deletion, insertion, or substitution can be also obtained by performing an in vitro mutation treatment or a site-directed mutagenesis treatment for the gene which codes for lysine decarboxylase having the amino acid sequence shown in SEQ ID NO:4. These mutation treatments can be performed in accordance with methods well-known to those skilled in the art as described below.

However, the gene, which codes for lysine decarboxylase having substitution, deletion, or insertion of one or a plurality of amino acid residues as referred to herein, includes those which originate from the "ldc gene" and can be regarded to be substantially the same as the ldc gene. It is not intended to extend the meaning to those genes having different origins. It is impossible to concretely prescribe a certain range of the "plurality". However, it will be readily understood by those skilled in the art that, for example, the cadA gene which codes for the protein different in not less than 200 amino acid residues from one having the amino acid sequence shown in SEQ ID NO:3 is different from the gene of the present invention, and the genes which code for proteins having equivalent lysine decarboxylase activity are included in the present invention even if they are different from one having the amino acid sequence shown in SEQ ID NO:3 with respect to two or three amino acid residues.

<2>Creation of microorganism belonging to the genus Escherichia with restrained expression of lysine decarboxylase gene

The microorganism belonging to the genus Escherichia of the present invention is a microorganism belonging to the genus Escherichia in which the lysine decarboxylase activity in cells is decreased or disappeared. The microorganism belonging to the genus Escherichia includes *Escherichia coli*. The lysine decarboxylase activity in cells is decreased or disappeared, for example, by restraining expression of any one of or both of the novel lysine decarboxylase gene (ldc) and the known cadA gene described above. Alternatively, the lysine decarboxylase activity in cells can be also decreased or disappeared by decreasing or disappearing the specific activities of lysine decarboxylase enzymes encoded by these genes, by modifying the structure of the enzymes.

The means for restraining expression of the ldc gene and the known cadA gene includes, for example, a method for restraining expression of the genes at a transcription level by causing substitution, deletion, insertion, addition, or inversion of one or a plurality of nucleotides in promoter sequences of these genes, and decreasing promoter activities (M. Rosenberg and D. Court, *Ann. Rev. Genetics* 13 (1979) p 319, and P. Youderian, S. Bouvier and M. Susskind, Cell 30 (1982) p.843–853). Alternatively, the expression of these genes can be restrained at a translation level by causing substitution, deletion, insertion, addition, or inversion of one or a plurality of nucleotides in a region between an SD sequence and an initiation codon (J. J. Dunn, E. Buzash-Pollert and F. W. Studier, *Proc. Nat. Acad. Sci. U.S.A.*, 75 (1978) p.2743). In addition, in order to decrease or disappear the specific activity of the lysine decarboxylase enzyme, a method is available, in which the coding region of the lysine decarboxylase gene is modified or destroyed by causing substitution, deletion, insertion, addition, or inversion of one or a plurality of nucleotides in a nucleotide sequence in the coding region.

The gene, on which nucleotide substitution, deletion, insertion, addition, or inversion is allowed to occur, may be ldc genes or cadA genes having substitution, deletion, or insertion of one or a plurality of amino acid residues which do not deteriorate the substantial activity of encoded lysine decarboxylase, in addition to the ldc gene or the cadA gene.

The method to cause nucleotide substitution, deletion insertion, addition, or inversion in the gene specifically includes a site-directed mutagenesis method (Kramer, W. and Frits, H. J., *Mothods in Enzymoloay*, 154, 350 (1987)), and a treatment method by using a chemical agent such as sodium hyposulfite and hydroxylamine (Shortle, D. and Nathans, D., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 270 (1978)).

The site-directed mutagenesis method is a method to use a synthetic oligonucleotide, which is a technique to enable introduction of optional substitution, deletion, insertion, addition, or inversion into an optional and limited nucleotide pair. In order to utilize this method, at first, a single strand is prepared by denaturing a plasmid having a cloned objective gene with a determined nucleotide sequence of DNA. Next, a synthetic oligonucleotide complementary to a portion intended to cause mutation is synthesized. However, in this procedure, the synthetic oligonucleotide is not allowed to have a completely complementary sequence, but it is designed to have optional nucleotide substitution, deletion, insertion, addition, or inversion. After that, the single strand DNA is annealed with the synthetic oligonucleotide having the optional nucleotide substitution, deletion, insertion, addition, or inversion. A complete double strand plasmid is synthesized by using T4 ligase and Klenow fragment of DNA polymerase I, which is introduced into competent cells of *Escherichia coli*. Some of transformants thus obtained have a plasmid containing a gene in which the optional nucleotide substitution, deletion, insertion, addition, or inversion is fixed. A recombinant PCR method (*PCR Technology*, Stockton press (1989)) may be mentioned as a similar method capable of introducing mutation into a gene to make modification or destruction.

The method to use the chemical agent is a method in which mutation having nucleotide substitution, deletion, insertion, addition, or inversion is randomly introduced into a DNA fragment by treating the DNA fragment containing an objective gene directly with sodium hyposulfite, hydroxylamine or the like.

Expression of the ldc gene and/or the cadA gene in cells can be restrained by substituting a normal gene on chromosome of a microorganism belonging to the genus Escherichia with the modified or destroyed gene obtained by the introduction of mutation as described above. The method for substituting the gene includes methods which utilize homologous recombination (*Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory press (1972); Matsuyama, S. and Mizushima, S., *J. Bacteriol.*, 162, 1196

(1985)). The homologous recombination is based on an ability generally possessed by the microorganism belonging to the genus Escherichia. When a plasmid or the like having homology to a sequence on chromosome is introduced into cells, recombination occurs at a certain frequency at a place of the sequence having the homology, and the whole of the introduced plasmid is incorporated on the chromosome. After that, if further recombination occurs at the place of the sequence having the homology on the chromosome, the plasmid falls off from the chromosome again. However, during this process, the gene with introduced mutation is occasionally fixed preferentially on the chromosome depending on the position at which recombination takes place, and an original normal gene falls off from the chromosome together with the plasmid. Selection of such microbial strains makes it possible to obtain a microbial strain in which the normal gene on the chromosome is substituted with the modified or destroyed gene obtained by the introduction of mutation having nucleotide substitution, deletion, insertion, addition, or inversion.

The microorganism belonging to the genus Escherichia to be subjected to the gene substitution is a microorganism having L-lysine productivity. The microorganism belonging to the genus Escherichia having L-lysine productivity, for example, a microbial strain of *Escherichia coli* can be obtained by applying a mutation treatment to a strain having no L-lysine productivity to give it resistance to a lysine analog such as S-(2-aminoethyl)-L-cysteine (hereinafter referred to as "AEC"). Methods for the mutation treatment include methods in which cells of *Escherichia coli* are subjected to a treatment with a chemical agent such as N-methyl-N'-nitro-N-nitrosoguanidine and nitrous acid, or a treatment with irradiation of ultraviolet light, radiation or the like. Such a microbial strain specifically includes *Escherichia coli* AJ13069 (FERM P-14690). This microbial strain was bred by giving AEC resistance to W3110 strain originating from *Escherichia coli* K-12. *Escherichia coli* AJ13069 was deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology (postal code:305, 1–3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) under an accession number of FERM P-14690 on Dec. 6, 1994, transferred to international deposition based on the Budapest Treaty on Sep. 29, 1995, and given an accession number of FERM BP-5252.

The microbial strain of *Escherichia coli* having L-lysine productivity can be also bred by introducing and enhancing DNA which carries genetic information relevant to L-lysine biosynthesis by means of the gene recombination technology. The gene to be introduced are genes which code for enzymes on the biosynthetic pathway of L-lysine, such as aspartokinase, dihydrodipicolinate synthetase, dihydrodipicolinate reductase, succinyldiaminopimelate transaminase, and succinyldiaminopimelate deacylase. In the case of a gene of the enzyme which undergoes feedback inhibition by L-lysine such as aspartokinase and dihydrodipicolinate synthetase, it is desirable to use a mutant type gene coding for an enzyme which is desensitized from such inhibition. In order to introduce and enhance the gene, a method is available, in which the gene is ligated with a vector autonomously replicable in cells of *Escherichia coli* to prepare recombinant DNA with which *Escherichia coli* is transformed. Alternatively, the gene can be also incorporated into chromosome of a host in accordance with a method to use transduction, transposon (Berg, D. E. and Berg, C. M., *Bio/Technol*, 1, 417 (1983)), Mu phage (Japanese Patent Laid-open No. 2-109985), or homologous recombination (*Experiments in Molecular Genetics*, Cold Spring Harbor Lab. (1972))

Other methods to obtain the microorganism belonging to the genus Escherichia with destroyed function of the gene include a method to cause genetic mutation by applying a treatment with a chemical agent such as N-methyl-N'-nitro-N-nitrosoguanidine and nitrous acid, or a treatment with irradiation of ultraviolet light, radiation or the like, to cells of the microorganism belonging to the genus Escherichia having the gene.

In Example described below, an *Escherichia coli* strain with destroyed function of the lysine decarboxylase gene was created by deleting a part of its coding region, and inserting a drug resistance gene instead of it to obtain a lysine decarboxylase gene which was used to substitute a lysine decarboxylase gene on chromosome of *Escherichia coli* in accordance with the method utilizing homologous recombination described above.

It is possible to restrain expression of any one of the novel lysine decarboxylase gene of the present invention and cadA gene, or restrain expression of both of them, in one microbial strain. Expression of the lysine decarboxylase gene may be restrained in the microorganism belonging to the genus Escherichia having L-lysine productivity, or L-lysine productivity may be given to the microorganism belonging to the genus Escherichia with restrained expression of the lysine decarboxylase gene in accordance with the method described above.

<3>Production of L-lysine by using microorganism belonging to the genus Escherichia with restrained expression of lysine decarboxylase gene

A considerable amount of L-lysine is produced and accumulated in a culture liquid by cultivating the microorganism belonging to the genus Escherichia with restrained expression of the lysine decarboxylase gene obtained as described above. The accumulation amount of L-lysine is increased only by restraining expression of the known cadA gene. However, it is more effective for increasing the accumulation amount of L-lysine to restrain expression of the novel lysine decarboxylase gene of the present invention. The most preferable result for L-lysine production is obtained by using a microbial strain in which expression of both of the cadA gene and the novel gene of the present invention is restrained.

The medium to be used for L-lysine production is an ordinary medium containing a carbon source, a nitrogen source, inorganic ions, and optionally other organic trace nutrient sources. As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose, and starch hydrolysate; alcohols such as glycerol and sorbitol; and organic acids such as fumaric acid, citric acid, and succinic acid. As the nitrogen source, it is possible to use inorganic ammonium salts such as ammonium sulfate, ammonium chloride, and ammonium phosphate; organic nitrogen sources such as soybean hydrolysate; ammonia gas; and aqueous ammonia. As the inorganic ions, potassium phosphate, magnesium sulfate, iron ion, manganese ion and so on are added in small amounts. Other than the above, it is desirable to contain vitamin B_1, yeast extract or the like in appropriate amounts as the organic trace nutrient sources.

Cultivation is preferably carried out under an aerobic condition for about 16–72 hours. The cultivation temperature is controlled at 30° C. to 45° C., and pH is controlled at 5–7 during cultivation. Inorganic or organic, acidic or alkaline substances, or ammonia gas or the like can be used for pH adjustment.

After completion of the cultivation, collection of L-lysine from a fermented liquor can be appropriately carried out by

combining an ordinary ion exchange resin method, a precipitation method, and other known methods

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a structure of a plasmid pUC6F5HH5 containing the novel lysine decarboxylase gene

FIG. 2 shows a structure of a temperature-sensitive plasmid pTS6F5HH5 containing the novel lysine decarboxylase gene, and construction of a plasmid pTS6F5HH5Cm in which a part of the gene is substituted with a fragment containing a chloramphenicol resistance gene.

FIG. 3 shows comparison of L-lysine-decomposing activities in a strain WC196 harboring a normal lysine decarboxylase gene, and strains WC196C, WC196L, and WC196LC with destroyed lysine decarboxylase genes

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will be more specifically explained below with reference to Examples.

EXAMPLE 1

(1) Cloning of novel lysine decarboxylase gene

Chromosomal DNA was extracted in accordance with an ordinary method from cells of W3110 strain of *Escherichia coli* K-12 obtained from National Institute of Genetics (Yata 1111, Mishima-shi, Shizuoka-ken, Japan). On the other hand, two synthetic DNA primers as shown in SEQ ID NOS:1 and 2 in Sequence Listing were synthesized in accordance with an ordinary method on the basis of the nucleotide sequence of the cadA gene (see SEQ ID NO:5) described in Meng, S. and Bennett, G. N. ,*J. Bacteriol.*, 174, 2659 (1992). They had sequences homologous to a 5'-terminal upstream portion and a 3'-terminal portion of the cadA gene respectively. The chromosomal DNA and the DNA primers were used to perform a PCR method in accordance with the method of Erlich et al. (*PCR Technology*, Stockton press (1989)). Thus a DNA fragment of 2.1 kbp containing almost all parts of the cadA gene was obtained. This fragment was labeled with Random Primer Labeling Kit (produced by Takara Shuzo) and [α-^{32}P]dCTP (produced by Amersham Japan) to prepare a probe for hybridization.

Next, hybridization was performed in accordance with an ordinary method (*Molecular Cloning* (2nd edition), Cold Spring Harbor Laboratory press (1989)) by using the prepared probe and *Escherichia coli*/Gene Mapping Membrane (produced by Takara Shuzo). A library of Kohara et al. (lambda phage library of *Escherichia coli* chromosomal DNA:see Kohara, Y. et al. *Cell*, 50, 495–508 (1987)) had been adsorbed to *Escherichia coli*/Gene Mapping Membrane. Lambda phage clones having sequences similar to the cadA gene were screened by weakening the condition for washing the probe (2×SSC, 55° C., 30 minutes), when the hybridization was performed. As a result, we succeeded in finding weak signals from three clones of E2B8, 6F5H, and 10F9, in addition to strong signals from clones containing the cadA gene region (21H11, 5G7). Insertion sequences of the three lambda phage clones of E2B8, 6F5H, and 10F9 continue on chromosome of *Escherichia coli* while overlapping with each other. Thus lambda phage DNA of 6F5H belonging to the library of Kohara et al. (Kohara, Y. et al. *Cell*, 50, 495–508 (1987)) was separated in accordance with an ordinary method, which was digested with various restriction enzymes to perform Southern blot hybridization by using the probe described above in accordance with a method similar to one described above. As a result, it was revealed that a sequence similar to the cadA gene was present in a DNA fragment of about 5 kbp obtained by digestion with HindIII.

Thus, the fragment of about 5 kbp obtained by digesting the lambda phage DNA of 6F5H with HindIII was ligated with a HindIII digest of a plasmid pUC19 (produced by Takara Shuzo) by using T4 DNA ligase. This reaction mixture was used to transform *Escherichia coli* JM109 (produced by Takara Shuzo) to obtain ampicillin-resistant strains grown on a complete plate medium (containing 10 g of polypeptone, 5 g of yeast extract, and 5 g of sodium chloride in 1 L of water) added with 50 mg/mL ampicillin. A microbial strain was obtained therefrom, which harbored a plasmid with insertion of the fragment of about 5 kbp obtained by digesting the lambda phage DNA of 6F5H with HindIII. A plasmid was extracted from cells thereof, and a plasmid pUC6F5HH5 was obtained. FIG. 1 shows a structure of the plasmid pUC6F5HH5.

Escherichia coli JM109/pUC6F5HH5 harboring this plasmid was designated as AJ13068, deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under an accession number of FERM P-14689 on Dec. 6, 1994, transferred to international deposition based on the Budapest Treaty on Sep. 29, 1995, and given an accession number of FERM BP-5251.

(2) Determination of nucleotide sequence of novel lysine decarboxylase gene

A nucleotide sequence of a region between restriction enzyme sites of ClaI and HindIII of obtained pUC6F5HH5 was determined in accordance with a method described in *Molecular Cloning* (2nd edition), Cold Spring Harbor Laboratory press (1989). As a result, it was revealed that the nucleotide sequence shown in SEQ ID NO:3 in Sequence Listing was encoded. This DNA sequence contains an open reading frame which codes for the amino acid sequence shown in SEQ ID NO:4 in Sequence Listing.

(3) Preparation of *Escherichia coli* having L-lysine productivity

Escherichia coli W3110 was cultivated at 37° C. for 4 hours in a complete medium (containing 10 g of polypeptone, 5 g of yeast extract, and 5 g of sodium chloride in 1 L of water) to obtain microbial cells which were subjected to a mutation treatment at 37° C. for 30 minutes in a solution of N-methyl-N'-nitro-N-nitrosoguanidine at a concentration of 200 μg/ml, washed, and then applied to a minimum plate medium (containing 7 g of disodium hydrogenphosphate, 3 g of potassium dihydrogenphosphate, 1 g of ammonium chloride, 0.5 g of sodium chloride, 5 g of glucose, 0.25 g of magnesium sulfate hepta-hydrate, and 15 g of agar in 1 L of water) added with 5 g/L of AEC. AEC-resistant strains were obtained by separating colonies appeared after cultivation at 37° C. for 48 hours. WC196 strain as one strain among them had L-lysine productivity. WC196 strain was designated as AJ13069, deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under an accession number of FERM P-14690 on Dec. 6, 1994, transferred to international deposition based on the Budapest Treaty on Sep. 29, 1995, and given an accession number of FERM BP-5252.

(4) Creation of WC196 strain with destroyed function of novel lysine decarboxylase gene

The fragment of about 5 kbp obtained by digesting the lambda phage DNA of 6F5H with HindIII described above

was ligated with a HindIII digest of a temperature-sensitive plasmid pMAN031 (Yasueda, H et al., *Appl. Microbiol. Biotechnol.*, 36, 211 (1991)) by using T4 DNA ligase. This reaction mixture was used to transform *Escherichia coli* JM109, followed by cultivation at 37° C. for 24 hours on a complete plate medium added with 50 mg/L of ampicillin to grow ampicillin-resistant strains. A microbial strain was obtained therefrom, which harbored a plasmid with insertion of the fragment of about 5 kbp obtained by digesting the lambda phage DNA of 6F5H with HindIII. A plasmid was extracted from cells of this strain, and a plasmid pTS6F5HH5 was obtained. The plasmid pTS6F5HH5 was digested with EcoRV to remove a DNA fragment of about 1 kbp. Next, T4 ligase was used to insert a fragment having a chloramphenicol resistance gene of about 1 kbp obtained by digesting pHSG399 (produced by Takara Shuzo) with AccI. Thus a plasmid pTS6F5HH5Cm was constructed. As a result of the operation described above, we succeeded in construction of the plasmid having a DNA fragment with destroyed function of the novel lysine decarboxylase gene. FIG. 2 shows a structure of the plasmid pTS6F5HH5, and the plasmid pTS6F5HH5Cm.

Next, a strain was created, in which the novel lysine decarboxylase gene on chromosome of WC196 strain was substituted with the DNA fragment with destroyed function of the novel lysine decarboxylase gene, in accordance with a general homologous recombination technique (Matsuyama, S. and Mizushima, S., *J Bacteriol.*, 162, 1196 (1985)) by utilizing the property of temperature sensitivity of the plasmid pTS6F5HH5Cm. Namely, WC196 strain was transformed with the plasmid pTS6F5HH5Cm to firstly obtain a strain which was resistant to ampicillin and resistant to chloramphenicol at 30° C. Next, this strain was used to obtain a strain which was resistant to ampicillin and resistant to chloramphenicol at 42° C. Further, this strain was used to obtain a strain which was sensitive to ampicillin and resistant to chloramphenicol at 30° C. Thus the strain as described above was created, in which the novel lysine decarboxylase gene on chromosome of WC196 strain was substituted with the DNA fragment with destroyed function of the novel lysine decarboxylase gene. This strain was designated as WC196L strain.

(5) Creation of WC196 strain and WC196L strain with deficiency of cadA gene

Escherichia coli, in which cadA as the known lysine decarboxylase gene is destroyed, is already known, including, for example, GNB10181 strain originating from *Escherichia coli* K-12 (see Auger, E. A. et al., *Mol. Microbiol.*, 3, 609 (1989); this microbial strain is available from, for example, *E. coli* Genetic Stock Center (Connecticut, USA)). It has been revealed that the region of the cadA gene is deficient in this microbial strain. Thus the character of cadA gene deficiency of GNB10181 strain was transduced into WC196 strain in accordance with a general method by using P1 phage (*A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press (1992)) to create WC196C strain. Deficiency of the cadA gene of WC196 strain was confirmed by Southern blot hybridization. In addition, WC196LC strain with deficiency of the cadA gene was created from WC196L strain in accordance with a method similar to one described above.

EXAMPLE 2

(1) Confirmation of L-lysine-decomposing activities of WC196, WC196C, WC196L, and WC196LC strains

The four created strains described above were cultivated at 37° C. for 17 hours by using a medium for L-lysine production (containing 40 g of glucose, 16 g of ammonium sulfate, 1 g of potassium dihydrogenphosphate, 2 g of yeast extract, 10 mg of manganese sulfate tetra-to penta-hydrate, and 10 mg of iron sulfate hepta-hydrate in 1 L of water; pH was adjusted to 7.0 with potassium hydroxide, and then 30 g of separately sterilized calcium carbonate was added). Recovered microbial cells were washed twice with a physiological saline solution, suspended in a medium for assaying L-lysine decomposition (containing 17 g of disodium hydrogenphosphate dodeca-hydrate, 3 g of potassium dihydrogenphosphate, 0.5 g of sodium chloride, and 10 g of L-lysine hydrochloride in 1 L of water), and cultivated at 37° C. for 31 hours.

FIG. 3 shows changes in remaining L-lysine amounts in culture liquids in accordance with the passage of time. The amount of L-lysine was quantitatively determined by using Biotech Analyzer AS-210 (produced by Asahi Chemical Industry). Significant decomposition of L-lysine was observed in WC196 strain. However, the decomposing activity was decreased a little in WC196C strain with deficiency of the cadA gene as the known lysine decarboxylase gene. Decomposition of L-lysine was not observed in WC196L and WC196LC strains with destroyed function of the novel lysine decarboxylase gene. Remaining L-lysine in the culture liquid decreased during a period up to about 3 hours of cultivation in any of the microbial strains. However, this phenomenon was caused by incorporation of L-lysine into microbial cells, and not caused by decomposition.

(2) Production of L-lysine by WC196, WC196C, WC196L, and WC196LC strains

The four strains described above were cultivated at 37° C. for 20 hours in the medium for L-lysine production described above. The amounts of L-lysine and cadaverine produced and accumulated in culture liquids were measured. The amount of L-lysine was quantitatively determined by using Biotech Analyzer AS-210 as described above. The amount of cadaverine was quantitatively determined by using high performance liquid chromatography.

Results are shown in Table 1. The accumulation of L-lysine was increased, and the accumulation of cadaverine as a decomposition product of L-lysine was decreased in WC196C strain with destruction of the cadA gene as compared with WC196 strain, and in WC196L strain with destroyed function of the novel lysine decarboxylase gene as compared with WC196 and WC196C strains. The accumulation of L-lysine was further increased, and the accumulation of cadaverine as a decomposition product of L-lysine was not detected in WC196LC strain with destroyed function of the both lysine decarboxylase genes.

TABLE 1

Microbial strain	L-lysine accumulation (g/L)	Cadaverine accumulation (g/L)
WC196	1.4	0.6
WC196C	1.9	0.4
WC196L	2.3	0.1
WC196LC	3.3	not detected

EXAMPLE 3

Escherichia coli WC196LC with disappeared L-lysine-decomposing activity was transformed with pUC6F5HH5 containing the novel lysine decarboxylase gene to obtain an ampicillin-resistant strain. WC196LC strain and WC196LC/pUC6F5HH5 strain were cultivated at 37° C. for 16 hours in

a medium for L-lysine production added with 5 g/L of L-lysine, and the amount of produced cadaverine was measured.

Results are shown in Table 2. WC196LC strain failed to convert L-lysine into cadaverine, while WC196LC/pUC6F5HH5 strain had an ability to convert L-lysine into cadaverine.

TABLE 2

Microbial strain	Production amount of cadaverine (g/L)
WC196LC	not detected
WC196LC/pUC6F5HH5	0.93

Industrial Applicability

The novel lysine decarboxylase gene of the present invention participates in decomposition of L-lysine in *Escherichia coli*. L-lysine can be produced inexpensively and efficiently by cultivating the bacterium belonging to the genus Escherichia having L-lysine productivity with restrained expression of the gene described above and/or the cadA gene.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 6

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGGATAACCA CACCGCGTCT 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: YES

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGAAGGATCA TATTGGCGTT 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3183 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

-continued

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia Coli
(B) STRAIN: W3110

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1005..3143

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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ATCGATTCTC TGACTGCGGT TAGCCGTCAG GATGAGAAAC TGGATATTAA CATCGATGAA      60

GAAGTGCATC GTCTGCGTGA AAAAAGCGTA GAACTGACAC GTAAAATCTT CGCCGATCTC     120

GGTGCATGGC AGATTGCGCA ACTGGCACGC CATCCACAGC GTCCTTATAC CCTGGATTAC     180

GTTCGCCTGG CATTTGATGA ATTTGACGAA CTGGCTGGCG ACCGCGCGTA TGCAGACGAT     240

AAAGCTATCG TCGGTGGTAT CGCCCGTCTC GATGGTCGTC CGGTGATGAT CATTGGTCAT     300

CAAAAAGGTC GTGAAACCAA AGAAAAAATT CGCCGTAACT TTGGTATGCC AGCGCCAGAA     360

GGTTACCGCA AAGCACTGCG TCTGATGCAA ATGGCTGAAC GCTTTAAGAT GCCTATCATC     420

ACCTTTATCG ACACCCCGGG GGCTTATCCT GGCGTGGGCG CAGAAGAGCG TGGTCAGTCT     480

GAAGCCATTG CACGCAACCT GCGTGAAATG TCTCGCCTCG GCGTACCGGT AGTTTGTACG     540

GTTATCGGTG AAGGTGGTTC TGGCGGTGCG CTGGCGATTG GCGTGGGCGA TAAAGTGAAT     600

ATGCTGCAAT ACAGCACCTA TTCCGTTATC TCGCCGGAAG GTTGTGCGTC CATTCTGTGG     660

AAGAGCGCCG ACAAAGCGCC GCTGGCGGCT GAAGCGATGG GTATCATTGC TCCGCGTCTG     720

AAAGAACTGA AACTGATCGA CTCCATCATC CCGGAACCAC TGGGTGGTGC TCACCGTAAC     780

CCGGAAGCGA TGGCGGCATC GTTGAAAGCG CAACTGCTGG CGGATCTGGC CGATCTCGAC     840

GTGTTAAGCA CTGAAGATTT AAAAAATCGT CGTTATCAGC GCCTGATGAG CTACGGTTAC     900

GCGTAATTCG CAAAAGTTCT GAAAAAGGGT CACTTCGGTG GCCCTTTTTT ATCGCCACGG     960

TTTGAGCAGG CTATGATTAA GGAAGGATTT TCCAGGAGGA ACAC ATG AAC ATC ATT     1016
                                                 Met Asn Ile Ile
                                                   1

GCC ATT ATG GGA CCG CAT GGC GTC TTT TAT AAA GAT GAG CCC ATC AAA      1064
Ala Ile Met Gly Pro His Gly Val Phe Tyr Lys Asp Glu Pro Ile Lys
  5                  10                  15                  20

GAA CTG GAG TCG GCG CTG GTG GCG CAA GGC TTT CAG ATT ATC TGG CCA      1112
Glu Leu Glu Ser Ala Leu Val Ala Gln Gly Phe Gln Ile Ile Trp Pro
                  25                  30                  35

CAA AAC AGC GTT GAT TTG CTG AAA TTT ATC GAG CAT AAC CCT CGA ATT      1160
Gln Asn Ser Val Asp Leu Leu Lys Phe Ile Glu His Asn Pro Arg Ile
              40                  45                  50

TGC GGC GTG ATT TTT GAC TGG GAT GAG TAC AGT CTC GAT TTA TGT AGC      1208
Cys Gly Val Ile Phe Asp Trp Asp Glu Tyr Ser Leu Asp Leu Cys Ser
          55                  60                  65

GAT ATC AAT CAG CTT AAT GAA TAT CTC CCG CTT TAT GCC TTC ATC AAC      1256
Asp Ile Asn Gln Leu Asn Glu Tyr Leu Pro Leu Tyr Ala Phe Ile Asn
      70                  75                  80

ACC CAC TCG ACG ATG GAT GTC AGC GTG CAG GAT ATG CGG ATG GCG CTC      1304
Thr His Ser Thr Met Asp Val Ser Val Gln Asp Met Arg Met Ala Leu
  85                  90                  95                 100

TGG TTT TTT GAA TAT GCG CTG GGG CAG GCG GAA GAT ATC GCC ATT CGT      1352
Trp Phe Phe Glu Tyr Ala Leu Gly Gln Ala Glu Asp Ile Ala Ile Arg
                 105                 110                 115

ATG CGT CAG TAC ACC GAC GAA TAT CTT GAT AAC ATT ACA CCG CCG TTC      1400
Met Arg Gln Tyr Thr Asp Glu Tyr Leu Asp Asn Ile Thr Pro Pro Phe
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                    120                 125                 130

ACG AAA GCC TTG TTT ACC TAC GTC AAA GAG CGG AAG TAC ACC TTT TGT      1448
Thr Lys Ala Leu Phe Thr Tyr Val Lys Glu Arg Lys Tyr Thr Phe Cys
        135                 140                 145

ACG CCG GGG CAT ATG GGC GGC ACC GCA TAT CAA AAA AGC CCG GTT GGC      1496
Thr Pro Gly His Met Gly Gly Thr Ala Tyr Gln Lys Ser Pro Val Gly
    150                 155                 160

TGT CTG TTT TAT GAT TTT TTC GGC GGG AAT ACT CTT AAG GCT GAT GTC      1544
Cys Leu Phe Tyr Asp Phe Phe Gly Gly Asn Thr Leu Lys Ala Asp Val
165                 170                 175                 180

TCT ATT TCG GTC ACC GAG CTT GGT TCG TTG CTC GAC CAC ACC GGG CCA      1592
Ser Ile Ser Val Thr Glu Leu Gly Ser Leu Leu Asp His Thr Gly Pro
                185                 190                 195

CAC CTG GAA GCG GAA GAG TAC ATC GCG CGG ACT TTT GGC GCG GAA CAG      1640
His Leu Glu Ala Glu Glu Tyr Ile Ala Arg Thr Phe Gly Ala Glu Gln
            200                 205                 210

AGT TAT ATC GTT ACC AAC GGA ACA TCG ACG TCG AAC AAA ATT GTG GGT      1688
Ser Tyr Ile Val Thr Asn Gly Thr Ser Thr Ser Asn Lys Ile Val Gly
        215                 220                 225

ATG TAC GCC GCG CCA TCC GGC AGT ACG CTG TTG ATC GAC CGC AAT TGT      1736
Met Tyr Ala Ala Pro Ser Gly Ser Thr Leu Leu Ile Asp Arg Asn Cys
    230                 235                 240

CAT AAA TCG CTG GCG CAT CTG TTG ATG ATG AAC GAT GTA GTG CCA GTC      1784
His Lys Ser Leu Ala His Leu Leu Met Met Asn Asp Val Val Pro Val
245                 250                 255                 260

TGG CTG AAA CCG ACG CGT AAT GCG TTG GGG ATT CTT GGT GGG ATC CCG      1832
Trp Leu Lys Pro Thr Arg Asn Ala Leu Gly Ile Leu Gly Gly Ile Pro
                265                 270                 275

CGC CGT GAA TTT ACT CGC GAC AGC ATC GAA GAG AAA GTC GCT GCT ACC      1880
Arg Arg Glu Phe Thr Arg Asp Ser Ile Glu Glu Lys Val Ala Ala Thr
            280                 285                 290

ACG CAA GCA CAA TGG CCG GTT CAT GCG GTG ATC ACC AAC TCC ACC TAT      1928
Thr Gln Ala Gln Trp Pro Val His Ala Val Ile Thr Asn Ser Thr Tyr
        295                 300                 305

GAT GGC TTG CTC TAC AAC ACC GAC TGG ATC AAA CAG ACG CTG GAT GTC      1976
Asp Gly Leu Leu Tyr Asn Thr Asp Trp Ile Lys Gln Thr Leu Asp Val
    310                 315                 320

CCG TCG ATT CAC TTC GAT TCT GCC TGG GTG CCG TAC ACC CAT TTT CAT      2024
Pro Ser Ile His Phe Asp Ser Ala Trp Val Pro Tyr Thr His Phe His
325                 330                 335                 340

CCG ATC TAC CAG GGT AAA AGT GGT ATG AGC GGC GAG CGT GTT GCG GGA      2072
Pro Ile Tyr Gln Gly Lys Ser Gly Met Ser Gly Glu Arg Val Ala Gly
                345                 350                 355

AAA GTG ATC TTC GAA ACG CAA TCG ACC CAC AAA ATG CTG GCG GCG TTA      2120
Lys Val Ile Phe Glu Thr Gln Ser Thr His Lys Met Leu Ala Ala Leu
            360                 365                 370

TCG CAG GCT TCG CTG ATC CAC ATT AAA GGC GAG TAT GAC GAA GAG GCC      2168
Ser Gln Ala Ser Leu Ile His Ile Lys Gly Glu Tyr Asp Glu Glu Ala
        375                 380                 385

TTT AAC GAA GCC TTT ATG ATG CAT ACC ACC ACC TCG CCC AGT TAT CCC      2216
Phe Asn Glu Ala Phe Met Met His Thr Thr Thr Ser Pro Ser Tyr Pro
    390                 395                 400

ATT GTT GCT TCG GTT GAG ACG GCG GCG GCG ATG CTG CGT GGT AAT CCG      2264
Ile Val Ala Ser Val Glu Thr Ala Ala Ala Met Leu Arg Gly Asn Pro
405                 410                 415                 420

GGC AAA CGG CTG ATT AAC CGT TCA GTA GAA CGA GCT CTG CAT TTT CGC      2312
Gly Lys Arg Leu Ile Asn Arg Ser Val Glu Arg Ala Leu His Phe Arg
                425                 430                 435

AAA GAG GTC CAG CGG CTG CGG GAA GAG TCT GAC GGT TGG TTT TTC GAT      2360
Lys Glu Val Gln Arg Leu Arg Glu Glu Ser Asp Gly Trp Phe Phe Asp
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                    440                 445                 450

ATC TGG CAA CCG CCG CAG GTG GAT GAA GCC GAA TGC TGG CCC GTT GCG  2408
Ile Trp Gln Pro Pro Gln Val Asp Glu Ala Glu Cys Trp Pro Val Ala
        455             460             465

CCT GGC GAA CAG TGG CAC GGC TTT AAC GAT GCG GAT GCC GAT CAT ATG  2456
Pro Gly Glu Gln Trp His Gly Phe Asn Asp Ala Asp Ala Asp His Met
    470             475             480

TTT CTC GAT CCG GTT AAA GTC ACT ATT TTG ACA CCG GGG ATG GAC GAG  2504
Phe Leu Asp Pro Val Lys Val Thr Ile Leu Thr Pro Gly Met Asp Glu
485             490             495             500

CAG GGC AAT ATG AGC GAG GAG GGG ATC CCG GCG GCG CTG GTA GCA AAA  2552
Gln Gly Asn Met Ser Glu Glu Gly Ile Pro Ala Ala Leu Val Ala Lys
                505             510             515

TTC CTC GAC GAA CGT GGG ATC GTA GTA GAG AAA ACC GGC CCT TAT AAC  2600
Phe Leu Asp Glu Arg Gly Ile Val Val Glu Lys Thr Gly Pro Tyr Asn
            520             525             530

CTG CTG TTT CTC TTT AGT ATT GGC ATC GAT AAA ACC AAA GCA ATG GGA  2648
Leu Leu Phe Leu Phe Ser Ile Gly Ile Asp Lys Thr Lys Ala Met Gly
        535             540             545

TTA TTG CGT GGG TTG ACG GAA TTC AAA CGC TCT TAC GAT CTC AAC CTG  2696
Leu Leu Arg Gly Leu Thr Glu Phe Lys Arg Ser Tyr Asp Leu Asn Leu
    550             555             560

CGG ATC AAA AAT ATG CTA CCC GAT CTC TAT GCA GAA GAT CCC GAT TTC  2744
Arg Ile Lys Asn Met Leu Pro Asp Leu Tyr Ala Glu Asp Pro Asp Phe
565             570             575             580

TAC CGC AAT ATG CGT ATT CAG GAT CTG GCA CAA GGG ATC CAT AAG CTG  2792
Tyr Arg Asn Met Arg Ile Gln Asp Leu Ala Gln Gly Ile His Lys Leu
                585             590             595

ATT CGT AAA CAC GAT CTT CCC GGT TTG ATG TTG CGG GCA TTC GAT ACT  2840
Ile Arg Lys His Asp Leu Pro Gly Leu Met Leu Arg Ala Phe Asp Thr
            600             605             610

TTG CCG GAG ATG ATC ATG ACG CCA CAT CAG GCA TGG CAA CGA CAA ATT  2888
Leu Pro Glu Met Ile Met Thr Pro His Gln Ala Trp Gln Arg Gln Ile
        615             620             625

AAA GGC GAA GTA GAA ACC ATT GCG CTG GAA CAA CTG GTC GGT AGA GTA  2936
Lys Gly Glu Val Glu Thr Ile Ala Leu Glu Gln Leu Val Gly Arg Val
    630             635             640

TCG GCA AAT ATG ATC CTG CCT TAT CCA CCG GGC GTA CCG CTG TTG ATG  2984
Ser Ala Asn Met Ile Leu Pro Tyr Pro Pro Gly Val Pro Leu Leu Met
645             650             655             660

CCT GGA GAA ATG CTG ACC AAA GAG AGC CGC ACA GTA CTC GAT TTT CTA  3032
Pro Gly Glu Met Leu Thr Lys Glu Ser Arg Thr Val Leu Asp Phe Leu
                665             670             675

CTG ATG CTT TGT TCC GTC GGG CAA CAT TAC CCC GGT TTT GAA ACG GAT  3080
Leu Met Leu Cys Ser Val Gly Gln His Tyr Pro Gly Phe Glu Thr Asp
            680             685             690

ATT CAC GGC GCG AAA CAG GAC GAA GAC GGC GTT TAC CGC GTA CGA GTC  3128
Ile His Gly Ala Lys Gln Asp Glu Asp Gly Val Tyr Arg Val Arg Val
        695             700             705

CTA AAA ATG GCG GGA TAACTTGCCA GAGCGGCTTC CGGGCGAGTA ACGTTCTGTT  3183
Leu Lys Met Ala Gly
    710
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 713 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

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(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Asn Ile Ile Ala Ile Met Gly Pro His Gly Val Phe Tyr Lys Asp
1 5 10 15

Glu Pro Ile Lys Glu Leu Glu Ser Ala Leu Val Ala Gln Gly Phe Gln
20 25 30

Ile Ile Trp Pro Gln Asn Ser Val Asp Leu Leu Lys Phe Ile Glu His
35 40 45

Asn Pro Arg Ile Cys Gly Val Ile Phe Asp Trp Asp Glu Tyr Ser Leu
50 55 60

Asp Leu Cys Ser Asp Ile Asn Gln Leu Asn Glu Tyr Leu Pro Leu Tyr
65 70 75 80

Ala Phe Ile Asn Thr His Ser Thr Met Asp Val Ser Val Gln Asp Met
85 90 95

Arg Met Ala Leu Trp Phe Phe Glu Tyr Ala Leu Gly Gln Ala Glu Asp
100 105 110

Ile Ala Ile Arg Met Arg Gln Tyr Thr Asp Glu Tyr Leu Asp Asn Ile
115 120 125

Thr Pro Pro Phe Thr Lys Ala Leu Phe Thr Tyr Val Lys Glu Arg Lys
130 135 140

Tyr Thr Phe Cys Thr Pro Gly His Met Gly Gly Thr Ala Tyr Gln Lys
145 150 155 160

Ser Pro Val Gly Cys Leu Phe Tyr Asp Phe Phe Gly Gly Asn Thr Leu
165 170 175

Lys Ala Asp Val Ser Ile Ser Val Thr Glu Leu Gly Ser Leu Leu Asp
180 185 190

His Thr Gly Pro His Leu Glu Ala Glu Glu Tyr Ile Ala Arg Thr Phe
195 200 205

Gly Ala Glu Gln Ser Tyr Ile Val Thr Asn Gly Thr Ser Thr Ser Asn
210 215 220

Lys Ile Val Gly Met Tyr Ala Ala Pro Ser Gly Ser Thr Leu Leu Ile
225 230 235 240

Asp Arg Asn Cys His Lys Ser Leu Ala His Leu Leu Met Met Asn Asp
245 250 255

Val Val Pro Val Trp Leu Lys Pro Thr Arg Asn Ala Leu Gly Ile Leu
260 265 270

Gly Gly Ile Pro Arg Arg Glu Phe Thr Arg Asp Ser Ile Glu Glu Lys
275 280 285

Val Ala Ala Thr Thr Gln Ala Gln Trp Pro Val His Ala Val Ile Thr
290 295 300

Asn Ser Thr Tyr Asp Gly Leu Leu Tyr Asn Thr Asp Trp Ile Lys Gln
305 310 315 320

Thr Leu Asp Val Pro Ser Ile His Phe Asp Ser Ala Trp Val Pro Tyr
325 330 335

Thr His Phe His Pro Ile Tyr Gln Gly Lys Ser Gly Met Ser Gly Glu
340 345 350

Arg Val Ala Gly Lys Val Ile Phe Glu Thr Gln Ser Thr His Lys Met
355 360 365

Leu Ala Ala Leu Ser Gln Ala Ser Leu Ile His Ile Lys Gly Glu Tyr
370 375 380

Asp Glu Glu Ala Phe Asn Glu Ala Phe Met Met His Thr Thr Thr Ser
385 390 395 400

Pro Ser Tyr Pro Ile Val Ala Ser Val Glu Thr Ala Ala Ala Met Leu
405 410 415

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Arg Gly Asn Pro Gly Lys Arg Leu Ile Asn Arg Ser Val Glu Arg Ala
420 425 430

Leu His Phe Arg Lys Glu Val Gln Arg Leu Arg Glu Glu Ser Asp Gly
435 440 445

Trp Phe Phe Asp Ile Trp Gln Pro Pro Gln Val Asp Glu Ala Glu Cys
450 455 460

Trp Pro Val Ala Pro Gly Glu Gln Trp His Gly Phe Asn Asp Ala Asp
465 470 475 480

Ala Asp His Met Phe Leu Asp Pro Val Lys Val Thr Ile Leu Thr Pro
485 490 495

Gly Met Asp Glu Gln Gly Asn Met Ser Glu Glu Gly Ile Pro Ala Ala
500 505 510

Leu Val Ala Lys Phe Leu Asp Glu Arg Gly Ile Val Val Glu Lys Thr
515 520 525

Gly Pro Tyr Asn Leu Leu Phe Leu Phe Ser Ile Gly Ile Asp Lys Thr
530 535 540

Lys Ala Met Gly Leu Leu Arg Gly Leu Thr Glu Phe Lys Arg Ser Tyr
545 550 555 560

Asp Leu Asn Leu Arg Ile Lys Asn Met Leu Pro Asp Leu Tyr Ala Glu
565 570 575

Asp Pro Asp Phe Tyr Arg Asn Met Arg Ile Gln Asp Leu Ala Gln Gly
580 585 590

Ile His Lys Leu Ile Arg Lys His Asp Leu Pro Gly Leu Met Leu Arg
595 600 605

Ala Phe Asp Thr Leu Pro Glu Met Ile Met Thr Pro His Gln Ala Trp
610 615 620

Gln Arg Gln Ile Lys Gly Glu Val Glu Thr Ile Ala Leu Glu Gln Leu
625 630 635 640

Val Gly Arg Val Ser Ala Asn Met Ile Leu Pro Tyr Pro Pro Gly Val
645 650 655

Pro Leu Leu Met Pro Gly Glu Met Leu Thr Lys Glu Ser Arg Thr Val
660 665 670

Leu Asp Phe Leu Leu Met Leu Cys Ser Val Gly Gln His Tyr Pro Gly
675 680 685

Phe Glu Thr Asp Ile His Gly Ala Lys Gln Asp Glu Asp Gly Val Tyr
690 695 700

Arg Val Arg Val Leu Lys Met Ala Gly
705 710

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2145 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli
(B) STRAIN: CS520

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1 2145

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(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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ATG AAC GTT ATT GCA ATA TTG AAT CAC ATG GGG GTT TAT TTT AAA GAA          48
Met Asn Val Ile Ala Ile Leu Asn His Met Gly Val Tyr Phe Lys Glu
 1               5                  10                  15

GAA CCC ATC CGT GAA CTT CAT CGC GCG CTT GAA CGT CTG AAC TTC CAG          96
Glu Pro Ile Arg Glu Leu His Arg Ala Leu Glu Arg Leu Asn Phe Gln
            20                  25                  30

ATT GTT TAC CCG AAC GAC CGT GAC GAC TTA TTA AAA CTG ATC GAA AAC         144
Ile Val Tyr Pro Asn Asp Arg Asp Asp Leu Leu Lys Leu Ile Glu Asn
        35                  40                  45

AAT GCG CGT CTG TGC GGC GTT ATT TTT GAC TGG GAT AAA TAT AAT CTC         192
Asn Ala Arg Leu Cys Gly Val Ile Phe Asp Trp Asp Lys Tyr Asn Leu
    50                  55                  60

GAG CTG TGC GAA GAA ATT AGC AAA ATG AAC GAG AAC CTG CCG TTG TAC         240
Glu Leu Cys Glu Glu Ile Ser Lys Met Asn Glu Asn Leu Pro Leu Tyr
65                  70                  75                  80

GCG TTC GCT AAT ACG TAT TCC ACT CTC GAT GTA AGC CTG AAT GAC CTG         288
Ala Phe Ala Asn Thr Tyr Ser Thr Leu Asp Val Ser Leu Asn Asp Leu
                85                  90                  95

CGT TTA CAG ATT AGC TTC TTT GAA TAT GCG CTG GGT GCT GCT GAA GAT         336
Arg Leu Gln Ile Ser Phe Phe Glu Tyr Ala Leu Gly Ala Ala Glu Asp
            100                 105                 110

CTG CCT CCG CTG ACT AAA GCA CTG TTT AAA TAT GTT CGT GAA GGT AAA         384
Leu Pro Pro Leu Thr Lys Ala Leu Phe Lys Tyr Val Arg Glu Gly Lys
        115                 120                 125

ATT GCT AAT AAG ATC AAG CAG ACC ACT GAC GAA TAT ATC AAC ACT ATT         432
Ile Ala Asn Lys Ile Lys Gln Thr Thr Asp Glu Tyr Ile Asn Thr Ile
    130                 135                 140

TAT ACT TTC TGT ACT CCT GGT CAC ATG GGC GGT ACT GCA TTC CAG AAA         480
Tyr Thr Phe Cys Thr Pro Gly His Met Gly Gly Thr Ala Phe Gln Lys
145                 150                 155                 160

AGC CCG GTA GGT AGC CTG TTC TAT GAT TTC TTT GGT CCG AAT ACC ATG         528
Ser Pro Val Gly Ser Leu Phe Tyr Asp Phe Phe Gly Pro Asn Thr Met
                165                 170                 175

AAA TCT GAT ATT TCC ATT TCA GTA TCT GAA CTG GGT TCT CTG CTG GAT         576
Lys Ser Asp Ile Ser Ile Ser Val Ser Glu Leu Gly Ser Leu Leu Asp
            180                 185                 190

CAC AGT GGT CCA CAC AAA GAA GCA GAA CAG TAT ATC GCT CGC GTC TTT         624
His Ser Gly Pro His Lys Glu Ala Glu Gln Tyr Ile Ala Arg Val Phe
        195                 200                 205

AAC GCA GAC CGC AGC TAC ATG GTG ACC AAC GGT ACT TCC ACT GCG AAC         672
Asn Ala Asp Arg Ser Tyr Met Val Thr Asn Gly Thr Ser Thr Ala Asn
    210                 215                 220

AAA ATT GTT GGT ATG TAC TCT GCT CCA GCA GGC AGC ACC ATT CTG ATT         720
Lys Ile Val Gly Met Tyr Ser Ala Pro Ala Gly Ser Thr Ile Leu Ile
225                 230                 235                 240

GAC CGT AAC TGC CAC AAA TCG CTG ACC CAC CTG ATG ATG ATG AGC GAT         768
Asp Arg Asn Cys His Lys Ser Leu Thr His Leu Met Met Met Ser Asp
                245                 250                 255

GTT ACG CCA ATC TAT TTC CGC CCG ACC CGT AAC GCT TAC GGT ATT CTT         816
Val Thr Pro Ile Tyr Phe Arg Pro Thr Arg Asn Ala Tyr Gly Ile Leu
            260                 265                 270

GGT GGT ATC CCA CAG AGT GAA TTC CAG CAC GCT ACC ATT GCT AAG CGC         864
Gly Gly Ile Pro Gln Ser Glu Phe Gln His Ala Thr Ile Ala Lys Arg
        275                 280                 285

GTG AAA GAA ACA CCA AAC GCA ACC TGG CCG GTA CAT GCT GTA ATT ACC         912
Val Lys Glu Thr Pro Asn Ala Thr Trp Pro Val His Ala Val Ile Thr
    290                 295                 300

AAC TCT ACC TAT GAT GGT CTG CTG TAC AAC ACC GAC TTC ATC AAG AAA         960
Asn Ser Thr Tyr Asp Gly Leu Leu Tyr Asn Thr Asp Phe Ile Lys Lys
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305                 310                 315                 320

ACA CTG GAT GTG AAA TCC ATC CAC TTT GAC TCC GCG TGG GTG CCT TAC  1008
Thr Leu Asp Val Lys Ser Ile His Phe Asp Ser Ala Trp Val Pro Tyr
                325                 330                 335

ACC AAC TTC TCA CCG ATT TAC GAA GGT AAA TGC GGT ATG AGC GGT GGC  1056
Thr Asn Phe Ser Pro Ile Tyr Glu Gly Lys Cys Gly Met Ser Gly Gly
            340                 345                 350

CGT GTA GAA GGG AAA GTG ATT TAC GAA ACC CAG TCC ACT CAC AAA CTG  1104
Arg Val Glu Gly Lys Val Ile Tyr Glu Thr Gln Ser Thr His Lys Leu
        355                 360                 365

CTG GCG GCG TTC TCT CAG GCT TCC ATG ATC CAC GTT AAA GGT GAC GTA  1152
Leu Ala Ala Phe Ser Gln Ala Ser Met Ile His Val Lys Gly Asp Val
    370                 375                 380

AAC GAA GAA ACC TTT AAC GAA GCC TAC ATG ATG CAC ACC ACC ACT TCT  1200
Asn Glu Glu Thr Phe Asn Glu Ala Tyr Met Met His Thr Thr Thr Ser
385                 390                 395                 400

CCG CAC TAC GGT ATC GTG GCG TCC ACT GAA ACC GCT GCG GCG ATG ATG  1248
Pro His Tyr Gly Ile Val Ala Ser Thr Glu Thr Ala Ala Ala Met Met
                405                 410                 415

AAA GGC AAT GCA GGT AAG CGT CTG ATC AAC GGT TCT ATT GAA CGT GCG  1296
Lys Gly Asn Ala Gly Lys Arg Leu Ile Asn Gly Ser Ile Glu Arg Ala
            420                 425                 430

ATC AAA TTC CGT AAA GAG ATC AAA CGT CTG AGA ACG GAA TCT GAT GGC  1344
Ile Lys Phe Arg Lys Glu Ile Lys Arg Leu Arg Thr Glu Ser Asp Gly
        435                 440                 445

TGG TTC TTT GAT GTA TGG CAG CCG GAT CAT ATC GAT ACG ACT GAA TGC  1392
Trp Phe Phe Asp Val Trp Gln Pro Asp His Ile Asp Thr Thr Glu Cys
    450                 455                 460

TGG CCG CTG CGT TCT GAC AGC ACC TGG CAC GGC TTC AAA AAC ATC GAT  1440
Trp Pro Leu Arg Ser Asp Ser Thr Trp His Gly Phe Lys Asn Ile Asp
465                 470                 475                 480

AAC GAG CAC ATG TAT CTT GAC CCG ATC AAA GTC ACC CTG CTG ACT CCG  1488
Asn Glu His Met Tyr Leu Asp Pro Ile Lys Val Thr Leu Leu Thr Pro
                485                 490                 495

GGG ATG GAA AAA GAC GGC ACC ATG AGC GAC TTT GGT ATT CCG GCC AGC  1536
Gly Met Glu Lys Asp Gly Thr Met Ser Asp Phe Gly Ile Pro Ala Ser
            500                 505                 510

ATC GTG GCG AAA TAC CTC GAC GAA CAT GGC ATC GTT GTT GAG AAA ACC  1584
Ile Val Ala Lys Tyr Leu Asp Glu His Gly Ile Val Val Glu Lys Thr
        515                 520                 525

GGT CCG TAT AAC CTG CTG TTC CTG TTC AGC ATC GGT ATC GAT AAG ACC  1632
Gly Pro Tyr Asn Leu Leu Phe Leu Phe Ser Ile Gly Ile Asp Lys Thr
    530                 535                 540

AAA GCA CTG AGC CTG CTG CGT GCT CTG ACT GAC TTT AAA CGT GCG TTC  1680
Lys Ala Leu Ser Leu Leu Arg Ala Leu Thr Asp Phe Lys Arg Ala Phe
545                 550                 555                 560

GAC CTG AAC CTG CGT GTG AAA AAC ATG CTG CCG TCT CTG TAT CGT GAA  1728
Asp Leu Asn Leu Arg Val Lys Asn Met Leu Pro Ser Leu Tyr Arg Glu
                565                 570                 575

GAT CCT GAA TTC TAT GAA AAC ATG CGT ATT CAG GAA CTG GCT CAG AAT  1776
Asp Pro Glu Phe Tyr Glu Asn Met Arg Ile Gln Glu Leu Ala Gln Asn
            580                 585                 590

ATC CAC AAA CTG ATT GTT CAC CAC AAT CTG CCG GAT CTG ATG TAT CGC  1824
Ile His Lys Leu Ile Val His His Asn Leu Pro Asp Leu Met Tyr Arg
        595                 600                 605

GCA TTT GAA GTG CTG CCG ACG ATG GTA ATG ACT CCG TAT GCT GCA TTC  1872
Ala Phe Glu Val Leu Pro Thr Met Val Met Thr Pro Tyr Ala Ala Phe
    610                 615                 620

CAG AAA GAG CTG CAC GGT ATG ACC GAA GAA GTT TAC CTC GAC GAA ATG  1920
Gln Lys Glu Leu His Gly Met Thr Glu Glu Val Tyr Leu Asp Glu Met
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625                 630                 635                 640

GTA GGT CGT ATT AAC GCC AAT ATG ATC CTT CCG TAC CCG CCG GGA GTT      1968
Val Gly Arg Ile Asn Ala Asn Met Ile Leu Pro Tyr Pro Pro Gly Val
                645                 650                 655

CCT CTG GTA ATG CCG GGT GAA ATG ATC ACC GAA GAA AGC CGT CCG GTT      2016
Pro Leu Val Met Pro Gly Glu Met Ile Thr Glu Glu Ser Arg Pro Val
            660                 665                 670

CTG GAG TTC CTG CAG ATG CTG TGT GAA ATC GGC GCT CAC TAT CCG GGC      2064
Leu Glu Phe Leu Gln Met Leu Cys Glu Ile Gly Ala His Tyr Pro Gly
        675                 680                 685

TTT GAA ACC GAT ATT CAC GGT GCA TAC CGT CAG GCT GAT GGC CGC TAT      2112
Phe Glu Thr Asp Ile His Gly Ala Tyr Arg Gln Ala Asp Gly Arg Tyr
    690                 695                 700

ACC GTT AAG GTA TTG AAA GAA GAA AGC AAA AAA                          2145
Thr Val Lys Val Leu Lys Glu Glu Ser Lys Lys
705                 710                 715
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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 715 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Met Asn Val Ile Ala Ile Leu Asn His Met Gly Val Tyr Phe Lys Glu
  1               5                  10                  15

Glu Pro Ile Arg Glu Leu His Arg Ala Leu Glu Arg Leu Asn Phe Gln
            20                  25                  30

Ile Val Tyr Pro Asn Asp Arg Asp Asp Leu Leu Lys Leu Ile Glu Asn
        35                  40                  45

Asn Ala Arg Leu Cys Gly Val Ile Phe Asp Trp Asp Lys Tyr Asn Leu
    50                  55                  60

Glu Leu Cys Glu Glu Ile Ser Lys Met Asn Glu Asn Leu Pro Leu Tyr
65                  70                  75                  80

Ala Phe Ala Asn Thr Tyr Ser Thr Leu Asp Val Ser Leu Asn Asp Leu
                85                  90                  95

Arg Leu Gln Ile Ser Phe Phe Glu Tyr Ala Leu Gly Ala Ala Glu Asp
            100                 105                 110

Leu Pro Pro Leu Thr Lys Ala Leu Phe Lys Tyr Val Arg Glu Gly Lys
        115                 120                 125

Ile Ala Asn Lys Ile Lys Gln Thr Thr Asp Glu Tyr Ile Asn Thr Ile
    130                 135                 140

Tyr Thr Phe Cys Thr Pro Gly His Met Gly Gly Thr Ala Phe Gln Lys
145                 150                 155                 160

Ser Pro Val Gly Ser Leu Phe Tyr Asp Phe Phe Gly Pro Asn Thr Met
                165                 170                 175

Lys Ser Asp Ile Ser Ile Ser Val Ser Glu Leu Gly Ser Leu Leu Asp
            180                 185                 190

His Ser Gly Pro His Lys Glu Ala Glu Gln Tyr Ile Ala Arg Val Phe
        195                 200                 205

Asn Ala Asp Arg Ser Tyr Met Val Thr Asn Gly Thr Ser Thr Ala Asn
    210                 215                 220

Lys Ile Val Gly Met Tyr Ser Ala Pro Ala Gly Ser Thr Ile Leu Ile
225                 230                 235                 240

Asp Arg Asn Cys His Lys Ser Leu Thr His Leu Met Met Met Ser Asp
```

-continued

				245								250								255											
Val	Thr	Pro	Ile	Tyr	Phe	Arg	Pro	Thr	Arg	Asn	Ala	Tyr	Gly	Ile	Leu																
			260					265					270																			
Gly	Gly	Ile	Pro	Gln	Ser	Glu	Phe	Gln	His	Ala	Thr	Ile	Ala	Lys	Arg																
		275					280					285																			
Val	Lys	Glu	Thr	Pro	Asn	Ala	Thr	Trp	Pro	Val	His	Ala	Val	Ile	Thr																
	290					295					300																				
Asn	Ser	Thr	Tyr	Asp	Gly	Leu	Leu	Tyr	Asn	Thr	Asp	Phe	Ile	Lys	Lys																
305					310					315					320																
Thr	Leu	Asp	Val	Lys	Ser	Ile	His	Phe	Asp	Ser	Ala	Trp	Val	Pro	Tyr																
				325					330					335																	
Thr	Asn	Phe	Ser	Pro	Ile	Tyr	Glu	Gly	Lys	Cys	Gly	Met	Ser	Gly	Gly																
			340					345					350																			
Arg	Val	Glu	Gly	Lys	Val	Ile	Tyr	Glu	Thr	Gln	Ser	Thr	His	Lys	Leu																
		355					360					365																				
Leu	Ala	Ala	Phe	Ser	Gln	Ala	Ser	Met	Ile	His	Val	Lys	Gly	Asp	Val																
	370					375					380																				
Asn	Glu	Glu	Thr	Phe	Asn	Glu	Ala	Tyr	Met	Met	His	Thr	Thr	Thr	Ser																
385					390					395					400																
Pro	His	Tyr	Gly	Ile	Val	Ala	Ser	Thr	Glu	Thr	Ala	Ala	Ala	Met	Met																
				405					410					415																	
Lys	Gly	Asn	Ala	Gly	Lys	Arg	Leu	Ile	Asn	Gly	Ser	Ile	Glu	Arg	Ala																
			420					425					430																			
Ile	Lys	Phe	Arg	Lys	Glu	Ile	Lys	Arg	Leu	Arg	Thr	Glu	Ser	Asp	Gly																
		435					440					445																				
Trp	Phe	Phe	Asp	Val	Trp	Gln	Pro	Asp	His	Ile	Asp	Thr	Thr	Glu	Cys																
	450					455					460																				
Trp	Pro	Leu	Arg	Ser	Asp	Ser	Thr	Trp	His	Gly	Phe	Lys	Asn	Ile	Asp																
465					470					475					480																
Asn	Glu	His	Met	Tyr	Leu	Asp	Pro	Ile	Lys	Val	Thr	Leu	Leu	Thr	Pro																
				485					490					495																	
Gly	Met	Glu	Lys	Asp	Gly	Thr	Met	Ser	Asp	Phe	Gly	Ile	Pro	Ala	Ser																
			500					505					510																			
Ile	Val	Ala	Lys	Tyr	Leu	Asp	Glu	His	Gly	Ile	Val	Val	Glu	Lys	Thr																
		515					520					525																				
Gly	Pro	Tyr	Asn	Leu	Leu	Phe	Leu	Phe	Ser	Ile	Gly	Ile	Asp	Lys	Thr																
	530					535					540																				
Lys	Ala	Leu	Ser	Leu	Leu	Arg	Ala	Leu	Thr	Asp	Phe	Lys	Arg	Ala	Phe																
545					550					555					560																
Asp	Leu	Asn	Leu	Arg	Val	Lys	Asn	Met	Leu	Pro	Ser	Leu	Tyr	Arg	Glu																
				565					570					575																	
Asp	Pro	Glu	Phe	Tyr	Glu	Asn	Met	Arg	Ile	Gln	Glu	Leu	Ala	Gln	Asn																
			580					585					590																			
Ile	His	Lys	Leu	Ile	Val	His	His	Asn	Leu	Pro	Asp	Leu	Met	Tyr	Arg																
		595					600					605																				
Ala	Phe	Glu	Val	Leu	Pro	Thr	Met	Val	Met	Thr	Pro	Tyr	Ala	Ala	Phe																
	610					615					620																				
Gln	Lys	Glu	Leu	His	Gly	Met	Thr	Glu	Glu	Val	Tyr	Leu	Asp	Glu	Met																
625					630					635					640																
Val	Gly	Arg	Ile	Asn	Ala	Asn	Met	Ile	Leu	Pro	Tyr	Pro	Pro	Gly	Val																
				645					650					655																	
Pro	Leu	Val	Met	Pro	Gly	Glu	Met	Ile	Thr	Glu	Glu	Ser	Arg	Pro	Val																
			660					665					670																			

-continued

Leu	Glu	Phe	Leu	Gln	Met	Leu	Cys	Glu	Ile	Gly	Ala	His	Tyr	Pro	Gly
		675					680					685			
Phe	Glu	Thr	Asp	Ile	His	Gly	Ala	Tyr	Arg	Gln	Ala	Asp	Gly	Arg	Tyr
	690					695					700				
Thr	Val	Lys	Val	Leu	Lys	Glu	Glu	Ser	Lys	Lys					
705					710					715					

What is claimed is:

1. An isolated nucleic acid molecule encoding a lysine decarboxylase, wherein the lysine decarboxylase comprises the amino acid sequence of SEQ ID NO:4.

2. The isolated nucleic acid molecule of claim 1 comprising a sequence corresponding to position 1005 through position 3143 of SEQ ID NO:3.

3. An isolated microorganism belonging to the genus Escherichia,

wherein the microorganism contains a mutant of a wild-type gene encoding a wild-type lysine decarboxylase;

the microorganism lacks the wild-type gene encoding the wild-type lysine decarboxylase;

the wild-type lysine decarboxylase comprises the amino acid sequence of SEQ ID NO:4; and

the mutant gene encodes no lysine decarboxylase having decarboxylating activity, the mutant gene encodes a mutant lysine decarboxylase having less decarboxylating activity than the wild-type lysine decarboxylase, or the mutant gene contains a mutation in a regulatory region causing the microorganism to produce less of the wild-type lysine decarboxylase than a microorganism containing the wild-type gene encoding the wild-type lysine decarboxylase.

4. The isolated microorganism of claim 3, wherein the mutant gene contains a mutation in a regulatory region causing the microorganism to produce less of the wild-type lysine decarboxylase than a microorganism containing the wild-type gene encoding the wild-type lysine decarboxylase.

5. The isolated microorganism of claim 3 belonging to the species *Escherichia coli*.

6. The isolated microorganism of claim 3, wherein the wild-type gene comprises a sequence corresponding to position 1005 through position 3143 of SEQ ID NO:3.

7. The isolated microorganism of claim 3, wherein the mutant gene encodes no lysine decarboxylase having decarboxylating activity.

8. The isolated microorganism of claim 3, wherein the mutant gene encodes a mutant lysine decarboxylase having less decarboxylating activity than the wild-type lysine decarboxylase.

9. The isolated microorganism of claim 3,

wherein the microorganism further contains a second mutant of a second wild-type gene encoding a second wild-type lysine decarboxylase;

the microorganism lacks the second wild-type gene encoding the second wild-type lysine decarboxylase;

the second wild-type lysine decarboxylase comprises the amino acid sequence of SEQ ID NO:6; and

the second mutant gene encodes no lysine decarboxylase having decarboxylating activity, the second mutant gene encodes a second mutant lysine decarboxylase having less decarboxylating activity than the second wild-type lysine decarboxylase, or the second mutant gene contains a mutation in a regulatory region causing the microorganism to produce less of the second wild-type lysine decarboxylase than a microorganism containing the second wild-type gene encoding the second wild-type lysine decarboxylase.

10. The isolated microorganism of claim 9, wherein the second mutant gene contains a mutation in a regulatory region causing the microorganism to produce less of the second wild-type lysine decarboxylase than a microorganism containing the second wild-type gene encoding the second wild-type lysine decarboxylase.

11. The isolated microorganism of claim 9, wherein the second mutant gene encodes no lysine decarboxylase having decarboxylating activity.

12. The isolated microorganism of claim 9, wherein the second mutant gene encodes a second mutant lysine decarboxylase having less decarboxylating activity than the second wild-type lysine decarboxylase.

13. A method for producing L-lysine, comprising:

(a) cultivating the microorganism of claim 3 in a liquid medium, thereby producing the L-lysine and accumulating the L-lysine in the liquid medium, and

(b) collecting the L-lysine produced and accumulated in step (a).

14. The method of claim 13, wherein the mutant gene contains a mutation in a regulatory region causing the microorganism to produce less of the wild-type lysine decarboxylase than a microorganism containing the wild-type gene encoding the wild-type lysine decarboxylase.

15. The method of claim 13, wherein the microorganism belongs to the species *Escherichia coli*.

16. The method of claim 13, wherein the wild-type gene comprises a sequence corresponding to position 1005 through position 3143 of SEQ ID NO:3.

17. The method of claim 13, wherein the mutant gene encodes no lysine decarboxylase having decarboxylating activity.

18. The method of claim 13, wherein the mutant gene encodes a mutant lysine decarboxylase having less decarboxylating activity than the wild-type lysine decarboxylase.

19. The method of claim 13,

wherein the microorganism further contains a second mutant gene of a second wild-type gene encoding a second wild-type lysine decarboxylase;

the microorganism lacks the second wild-type gene encoding the second wild-type lysine decarboxylase;

the second wild-type lysine decarboxylase comprises the amino acid sequence of SEQ ID NO:6; and

the second mutant gene encodes no lysine decarboxylase having decarboxylating activity, the second mutant gene encodes a second mutant lysine decarboxylase having less decarboxylating activity than the second wild-type lysine decarboxylase, or the second mutant gene contains a mutation in a regulatory region causing the microorganism to produce less of the second wild-type lysine decarboxylase than a microorganism containing the second wild-type gene encoding the second wild-type lysine decarboxylase

20 The method of claim **19**, wherein the second mutant gene contains a mutation in a regulatory region causing the microorganism to produce less of the second wild-type lysine decarboxylase than a microorganism containing the second wild-type gene encoding the second wild-type lysine decarboxylase.

21. The method of claim **19**, wherein the second mutant gene encodes no lysine decarboxylase having decarboxylating activity

22. The method of claim **19**, wherein the second mutant gene encodes a second mutant lysine decarboxylase having less decarboxylating activity than the second wild-type lysine decarboxylase.

* * * * *

EXHIBIT B

US006040160A

United States Patent [19]

Kojima et al.

[11] Patent Number: 6,040,160

[45] Date of Patent: Mar. 21, 2000

[54] METHOD OF PRODUCING L-LYSINE BY FERMENTATION

[75] Inventors: Hiroyuki Kojima; Yuri Ogawa; Kazue Kawamura; Konosuke Sano, all of Kawasaki, Japan

[73] Assignee: Ajinomoto Co., Inc., Tokyo, Japan

[21] Appl. No.: 08/648,010

[22] PCT Filed: Nov. 28, 1994

[86] PCT No.: PCT/JP94/01994

§ 371 Date: May 29, 1996

§ 102(e) Date: May 29, 1996

[87] PCT Pub. No.: WO95/16042

PCT Pub. Date: Jun. 15, 1995

[30] Foreign Application Priority Data

Dec. 8, 1993 [JP] Japan 5-308397

[51] Int. Cl.[7] C12P 13/08; C12N 1/20; C12N 15/00; C07H 21/04

[52] U.S. Cl. 435/115; 435/252.3; 435/254.11; 435/320.1; 435/325; 536/23.2

[58] Field of Search 435/115, 252.3, 435/254.11, 320.1, 325; 536/23.2

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Primary Examiner—Bradley Sisson
Assistant Examiner—Einar Stole
Attorney, Agent, or Firm—Oblon, Spivak, McClelland, Maier & Neustadt, P.C.

[57] ABSTRACT

A bacterium belonging to the genus Escherichia, which is transformed by introducing, into its cells, a DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus Escherichia having mutation to desensitize feedback inhibition by L-lysine and a DNA coding for an aspartokinase III originating from a bacterium belonging to the genus Escherichia having mutation to desensitize feedback inhibition by L-lysine; preferably a bacterium belonging to the genus Escherichia in which a dihydrodipicolinate reductase gene and a diaminopimelate dehydrogenase gene originating from *Brevibacterium lactofermentum* (or a succinyldiaminopimelate transaminase gene and a succinyldiaminopimelate deacylase gene) are further enhanced, is cultivated in an appropriate medium, L-lysine is produced and accumulated in a culture thereof, and L-lysine is collected from the culture.

22 Claims, 18 Drawing Sheets

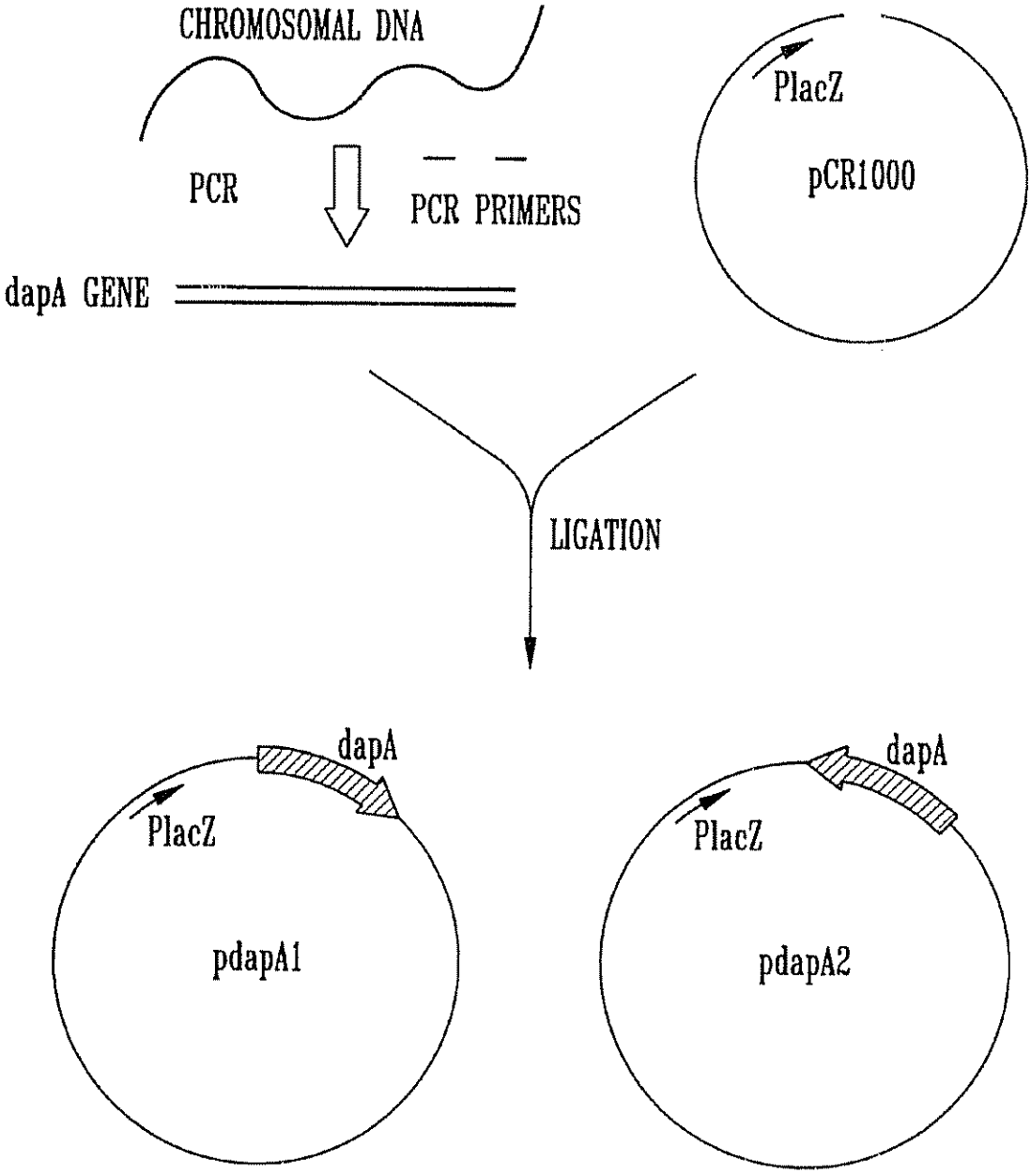
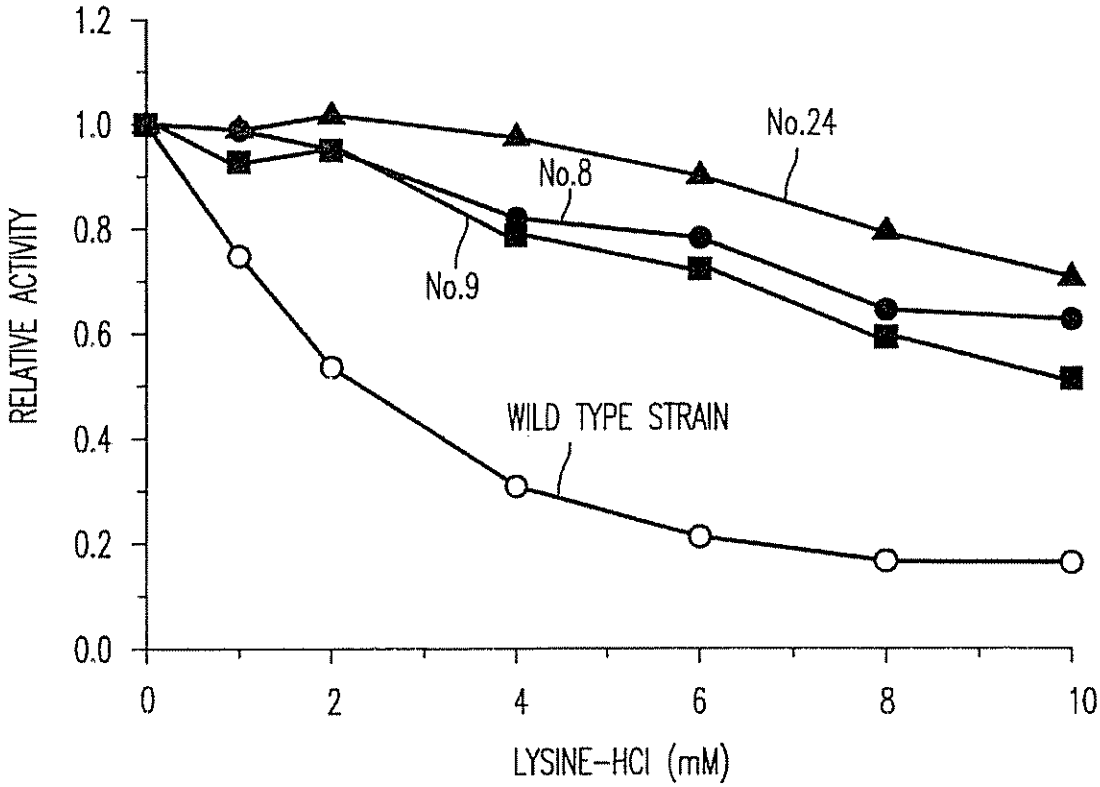

CHROMOSOMAL DNA
PCR
PCR PRIMERS
dapA GENE
PlacZ
pCR1000
LIGATION
dapA
PlacZ
pdapA1
dapA
PlacZ
pdapA2

FIG. 1

FIG. 2


1.2
1.0
0.8
0.6
0.4
0.2
0.0
RELATIVE ACTIVITY
No.24
No.8
No.9
WILD TYPE STRAIN
0
2
4
6
8
10
LYSINE-HCl (mM)

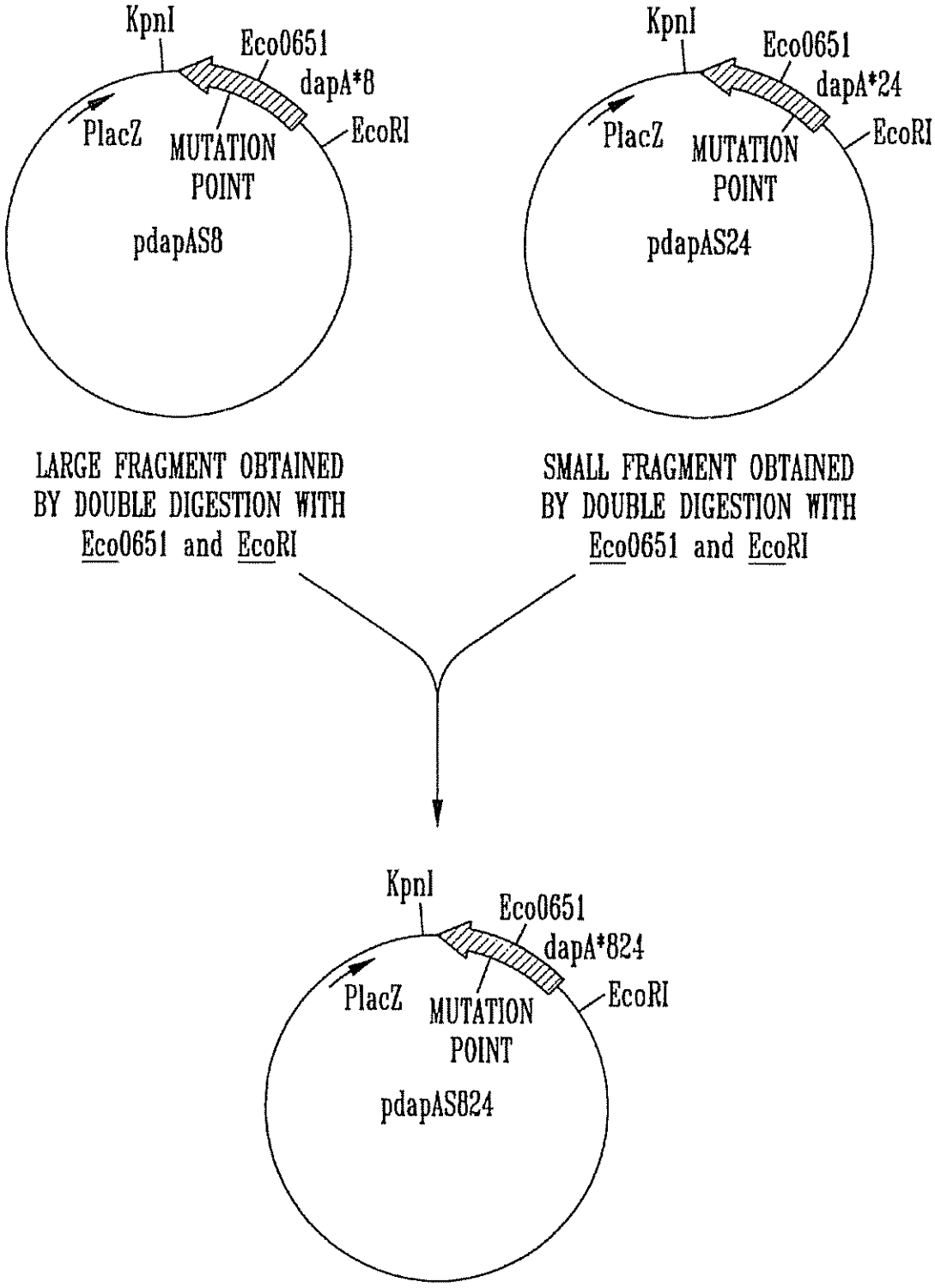

KpnI
Eco0651
dapA*8
PlacZ
MUTATION POINT
EcoRI
pdapAS8
KpnI
Eco0651
dapA*24
PlacZ
MUTATION POINT
EcoRI
pdapAS24
LARGE FRAGMENT OBTAINED BY DOUBLE DIGESTION WITH Eco0651 and EcoRI
SMALL FRAGMENT OBTAINED BY DOUBLE DIGESTION WITH Eco0651 and EcoRI
KpnI
Eco0651
dapA*824
PlacZ
MUTATION POINT
EcoRI
pdapAS824

FIG. 3

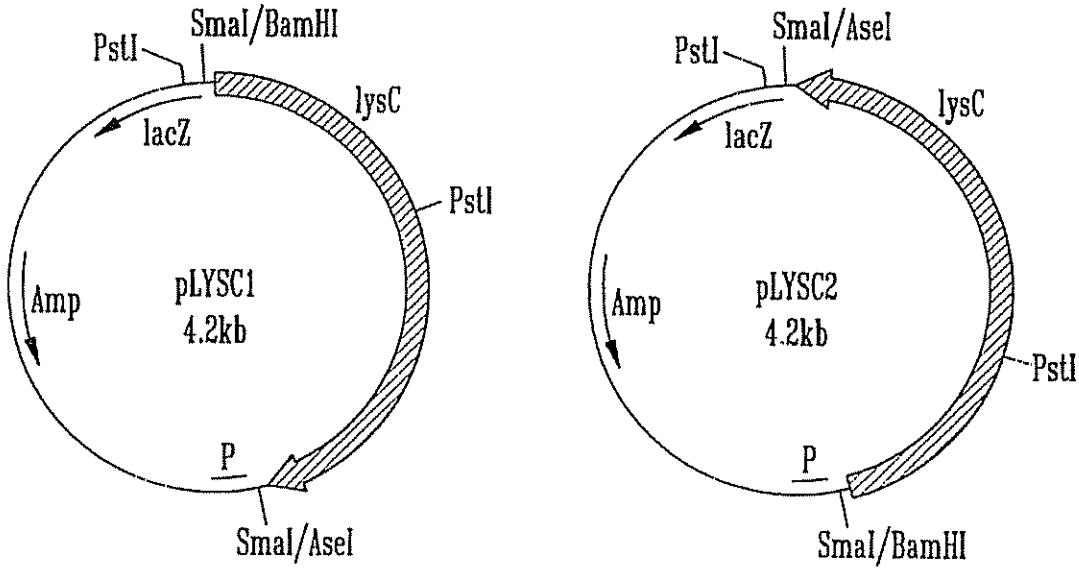
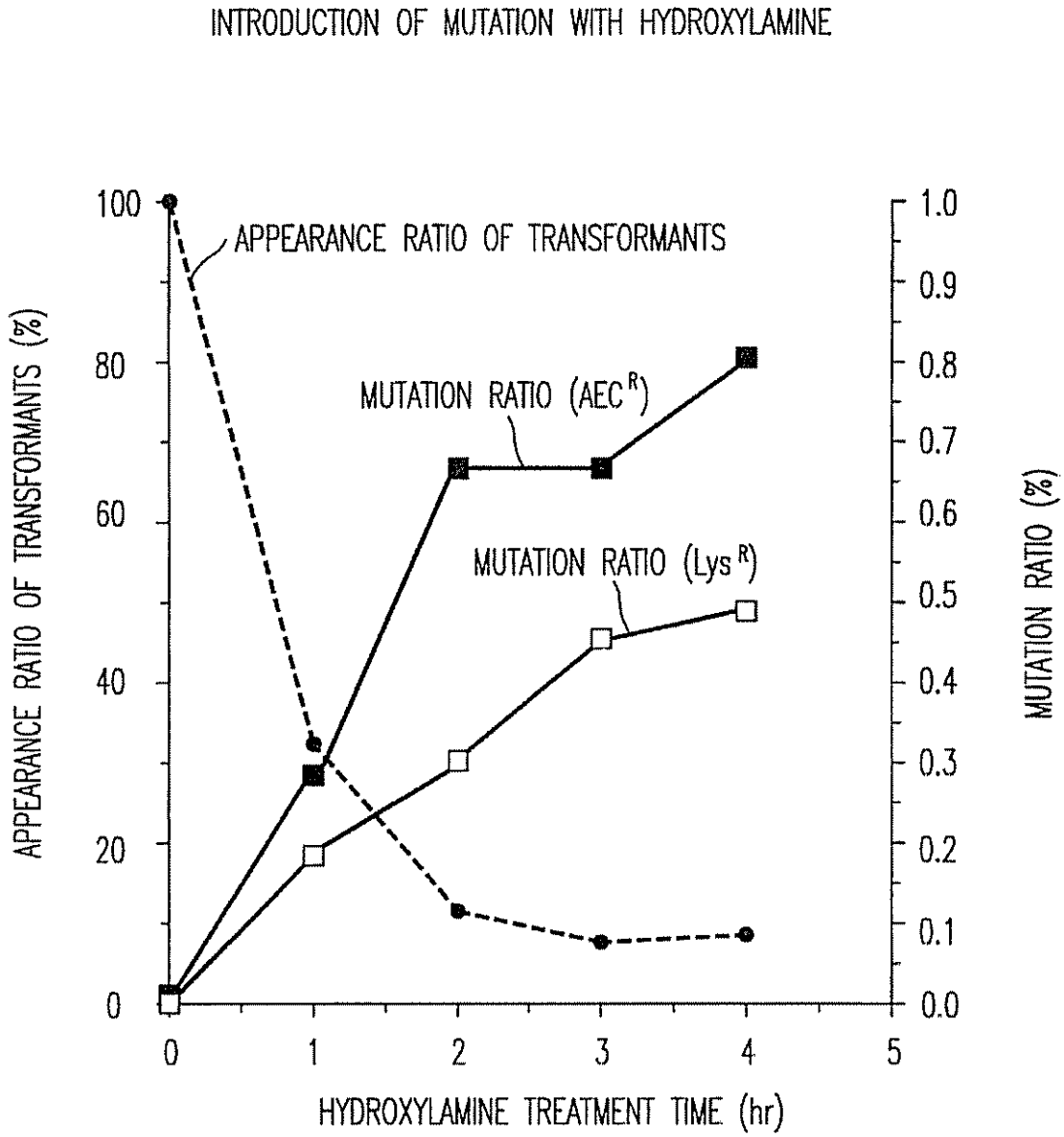

PstI
SmaI/BamHI
lysC
lacZ
PstI
Amp
pLYSC1
4.2kb
P
SmaI/AseI
PstI
SmaI/AseI
lysC
lacZ
Amp
pLYSC2
4.2kb
PstI
P
SmaI/BamHI

FIG. 4

FIG. 5


INTRODUCTION OF MUTATION WITH HYDROXYLAMINE
APPEARANCE RATIO OF TRANSFORMANTS
MUTATION RATIO (AEC R)
MUTATION RATIO (Lys R)
APPEARANCE RATIO OF TRANSFORMANTS (%)
MUTATION RATIO (%)
HYDROXYLAMINE TREATMENT TIME (hr)
100
80
60
40
20
0
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5

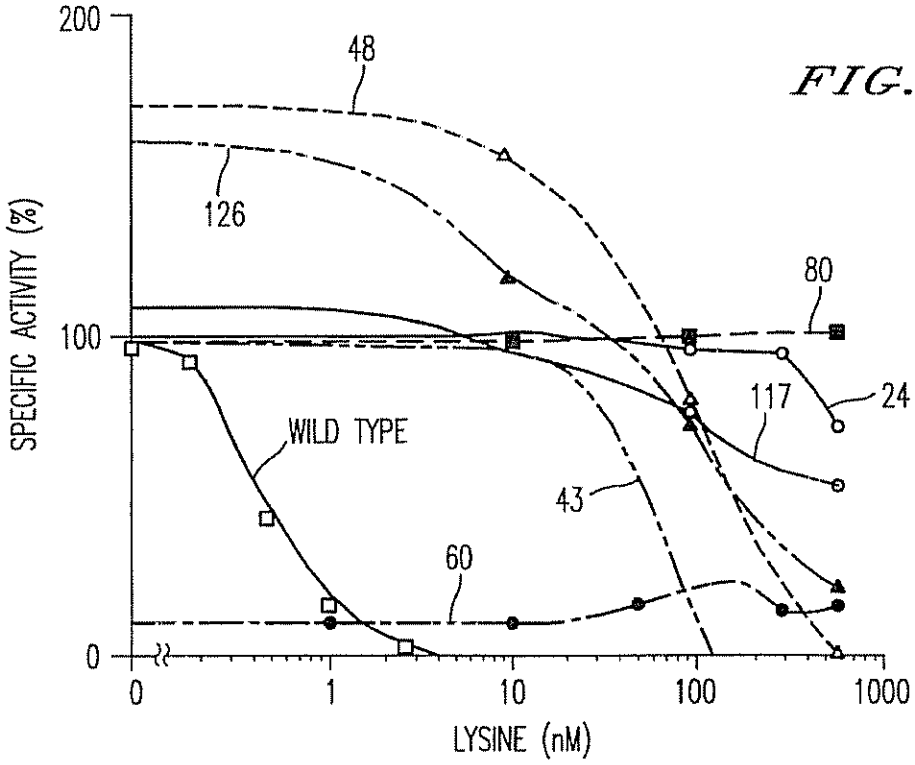

200
100
0
SPECIFIC ACTIVITY (%)
48
126
80
117
24
WILD TYPE
43
60
0
1
10
100
1000
LYSINE (nM)

FIG. 6A

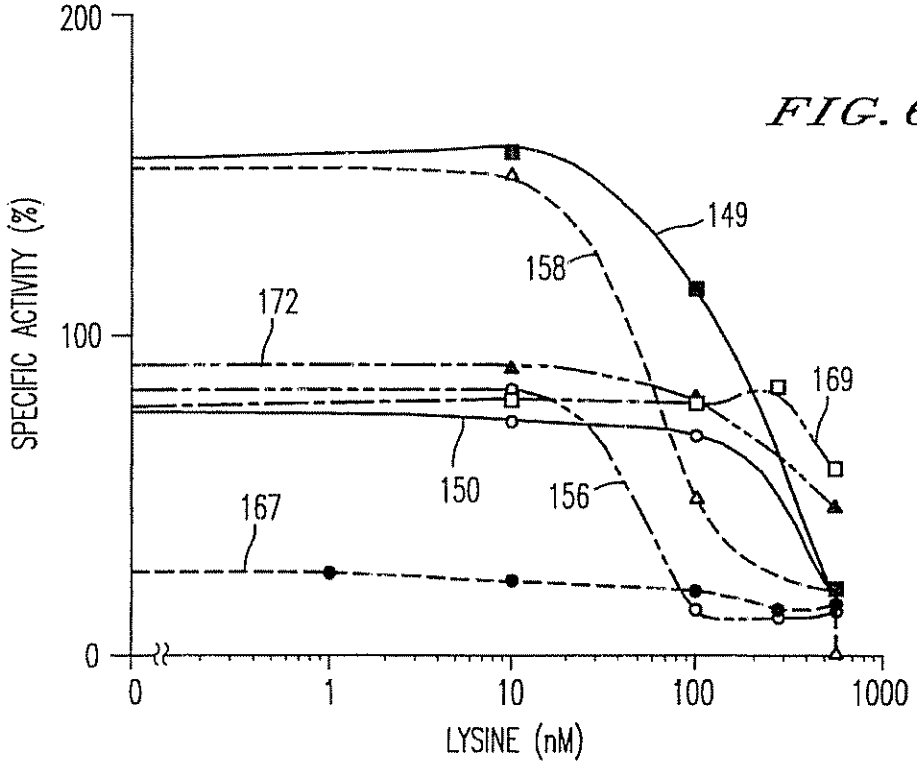

200
100
0
SPECIFIC ACTIVITY (%)
149
158
172
169
150
156
167
0
1
10
100
1000
LYSINE (nM)

FIG. 6B

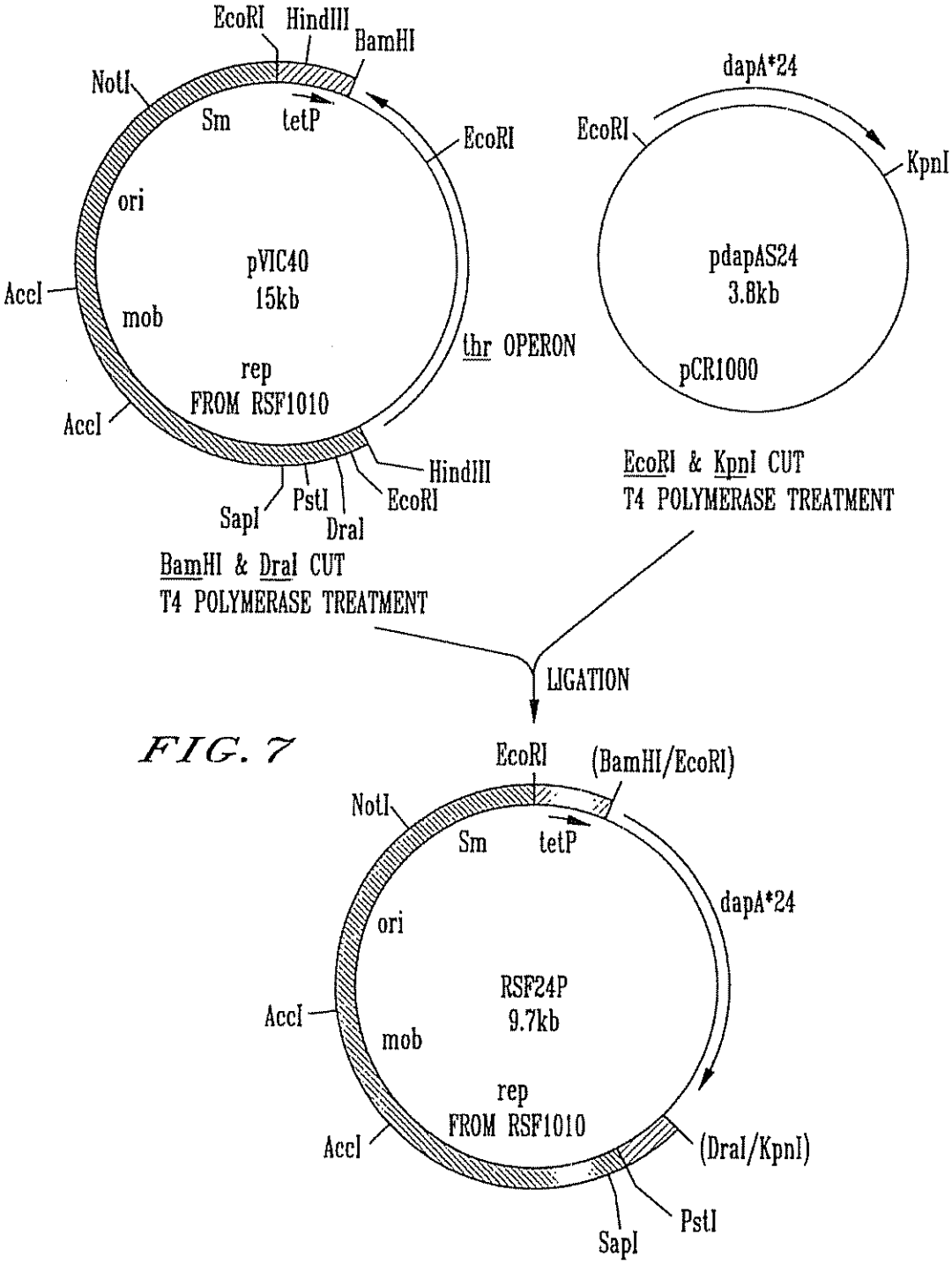

EcoRI
HindIII
BamHI
NotI
Sm
tetP
EcoRI
ori
pVIC40
15kb
AccI
mob
thr OPERON
rep
FROM RSF1010
AccI
HindIII
SapI
PstI
EcoRI
DraI
BamHI & DraI CUT
T4 POLYMERASE TREATMENT
dapA*24
EcoRI
KpnI
pdapAS24
3.8kb
pCR1000
EcoRI & KpnI CUT
T4 POLYMERASE TREATMENT
LIGATION
EcoRI
(BamHI/EcoRI)
NotI
Sm
tetP
dapA*24
ori
RSF24P
9.7kb
AccI
mob
rep
FROM RSF1010
AccI
(DraI/KpnI)
PstI
SapI

FIG. 7

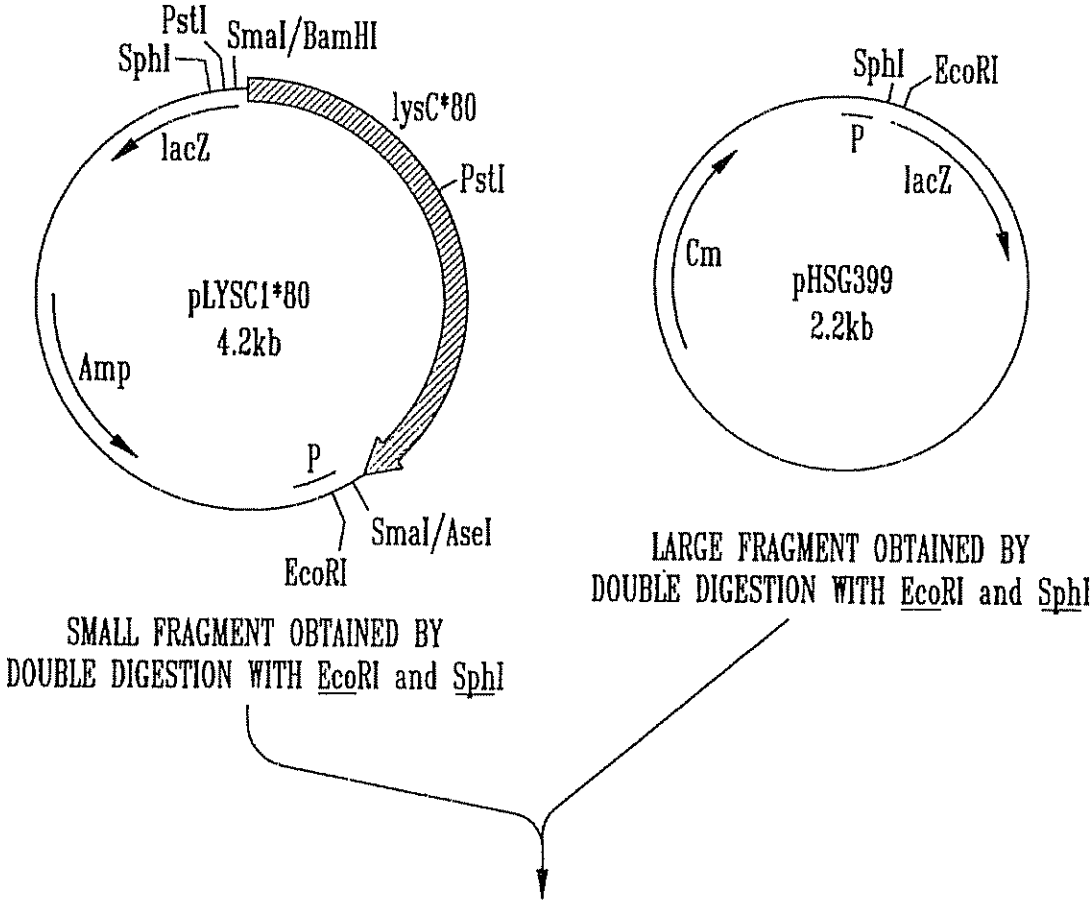
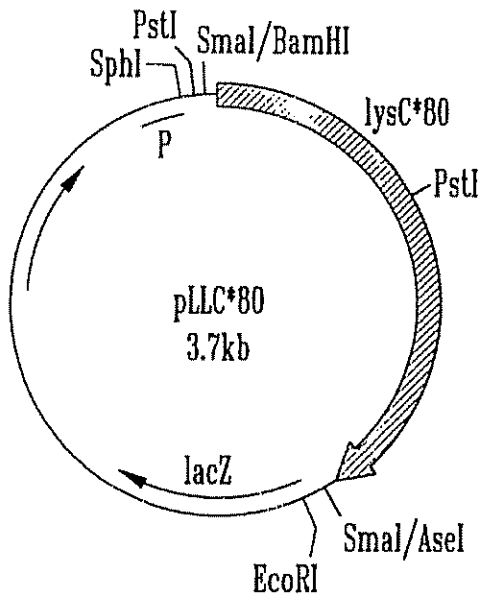

PstI
SmaI/BamHI
SphI
lysC*80
lacZ
PstI
pLYSC1*80
4.2kb
Amp
P
SmaI/AseI
EcoRI
SphI
EcoRI
P
lacZ
Cm
pHSG399
2.2kb
SMALL FRAGMENT OBTAINED BY
DOUBLE DIGESTION WITH EcoRI and SphI
LARGE FRAGMENT OBTAINED BY
DOUBLE DIGESTION WITH EcoRI and SphI

FIG. 8


PstI
SmaI/BamHI
SphI
P
lysC*80
PstI
pLLC*80
3.7kb
lacZ
SmaI/AseI
EcoRI

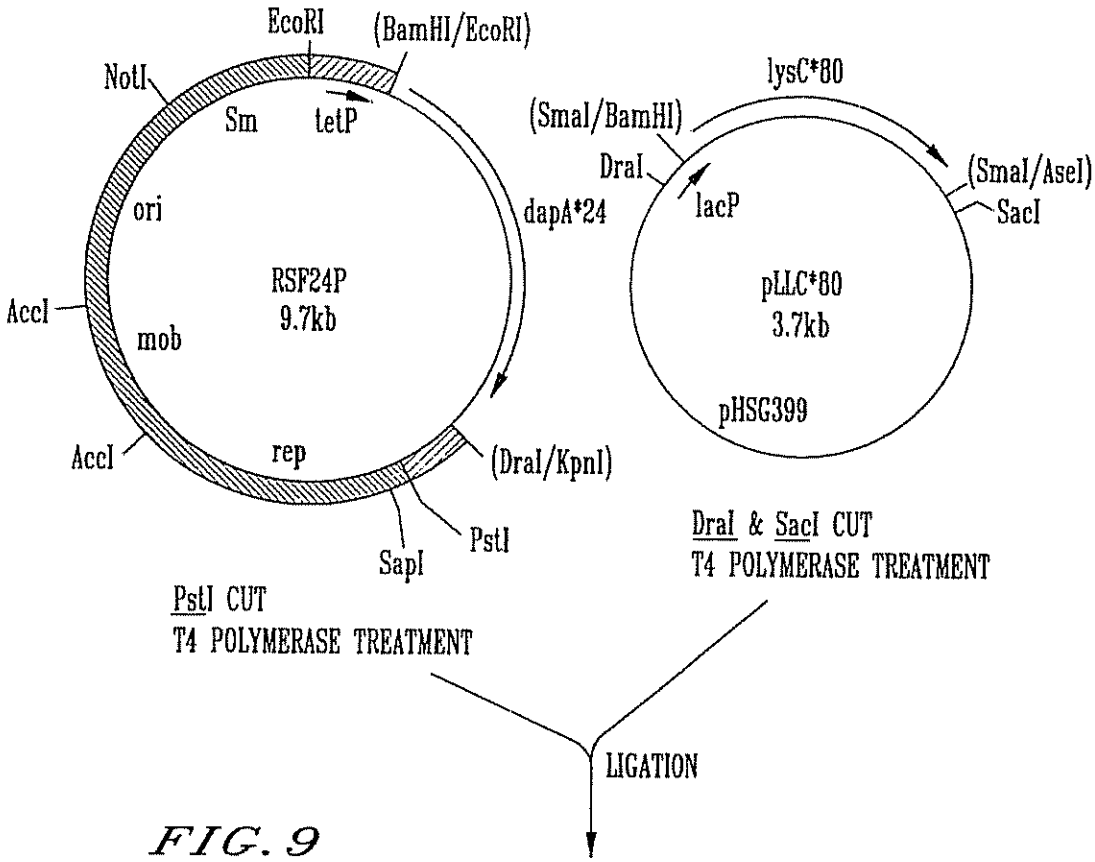
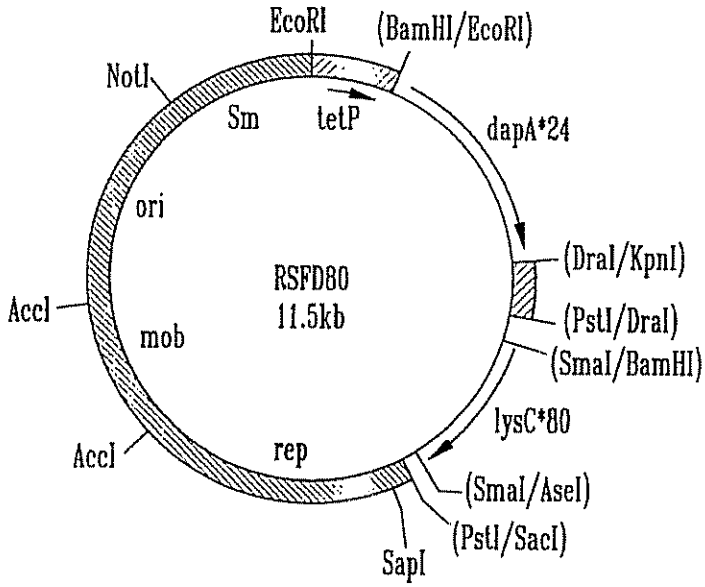

EcoRI
(BamHI/EcoRI)
NotI
Sm
tetP
ori
dapA*24
RSF24P
9.7kb
AccI
mob
AccI
rep
(DraI/KpnI)
PstI
SapI
lysC*80
(SmaI/BamHI)
DraI
lacP
(SmaI/AseI)
SacI
pLLC*80
3.7kb
pHSC399
DraI & SacI CUT
T4 POLYMERASE TREATMENT
PstI CUT
T4 POLYMERASE TREATMENT
LIGATION

FIG. 9


EcoRI
(BamHI/EcoRI)
NotI
Sm
tetP
dapA*24
ori
(DraI/KpnI)
RSFD80
11.5kb
AccI
(PstI/DraI)
mob
(SmaI/BamHI)
lysC*80
AccI
rep
(SmaI/AseI)
(PstI/SacI)
SapI

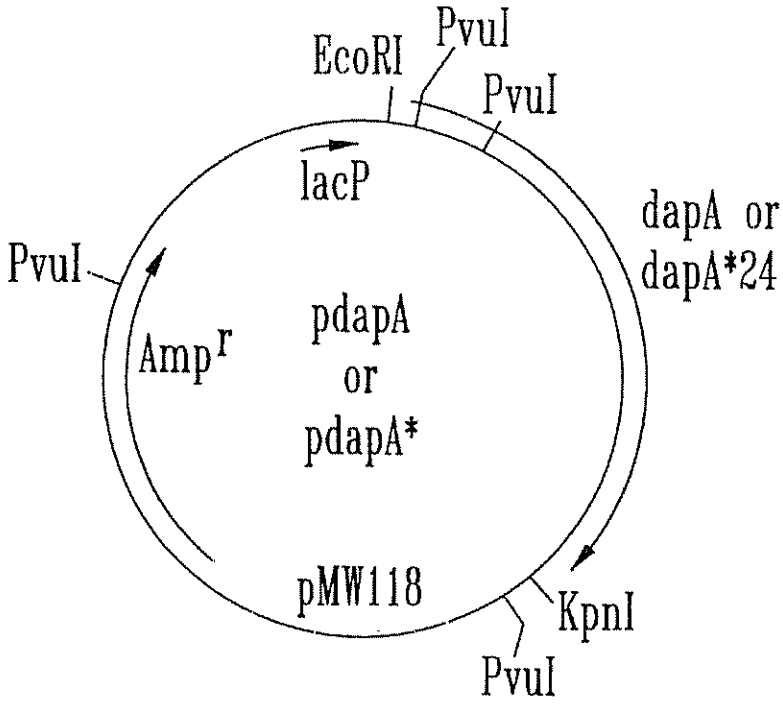

EcoRI
PvuI
PvuI
lacP
dapA or
dapA*24
PvuI
Amp^r
pdapA
or
pdapA*
pMW118
KpnI
PvuI

FIG. 10

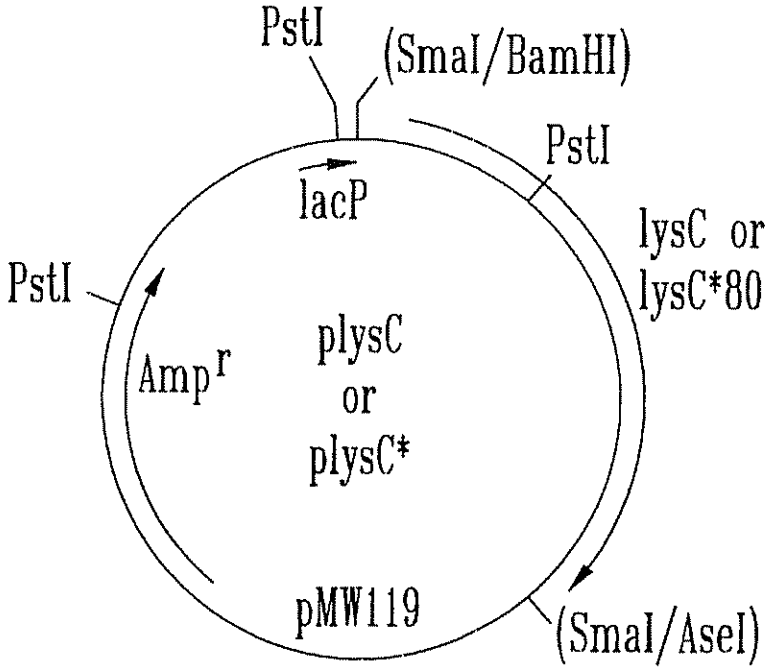
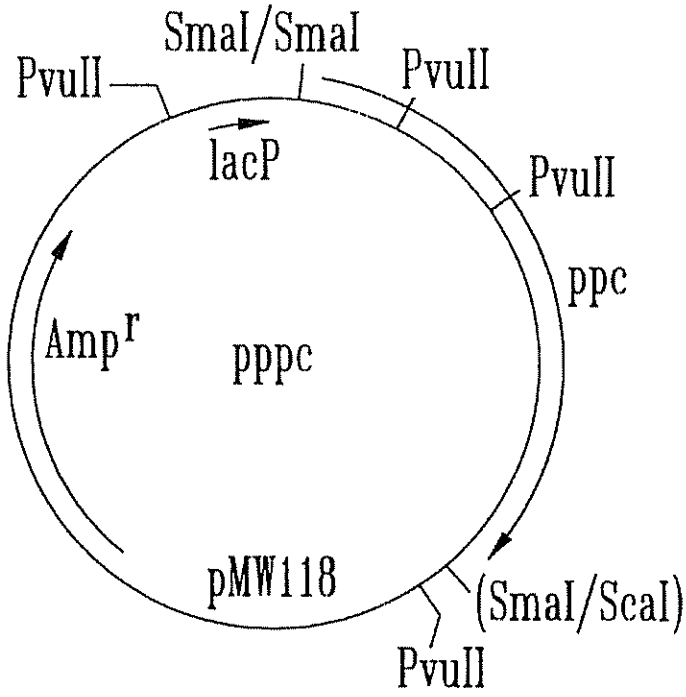

PstI
(SmaI/BamHI)
PstI
lacP
lysC or
lysC*80
PstI
Amp^r
plysC
or
plysC*
pMW119
(SmaI/AseI)

FIG. 11


SmaI/SmaI
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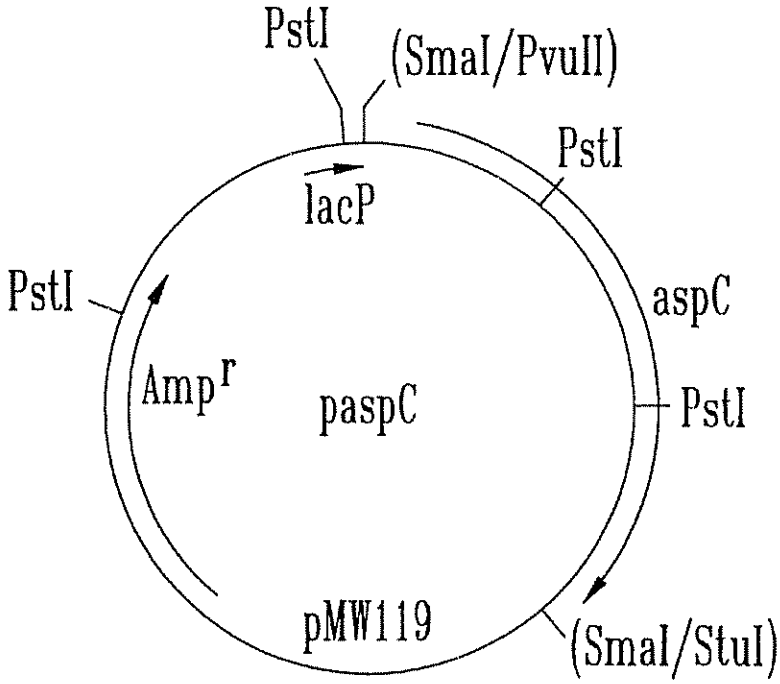
PvuII
lacP
PvuII
ppc
Amp^r
pppc
pMW118
(SmaI/ScaI)
PvuII

FIG. 12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(SmaI/PvuII)
PstI
lacP
PstI
aspC
Amp^r
paspC
PstI
pMW119
(SmaI/StuI)

FIG. 13

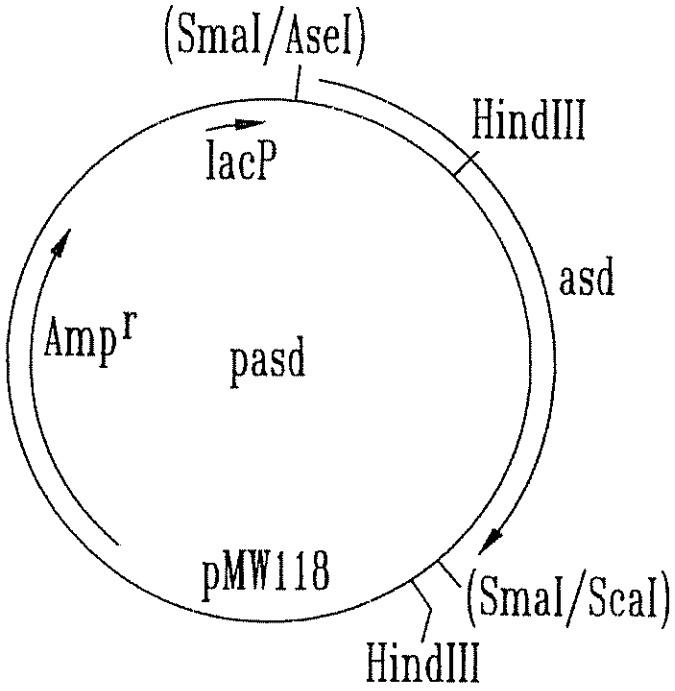

(SmaI/AseI)
HindIII
lacP
asd
Amp^r
pasd
pMW118
(SmaI/ScaI)
HindIII

FIG. 14

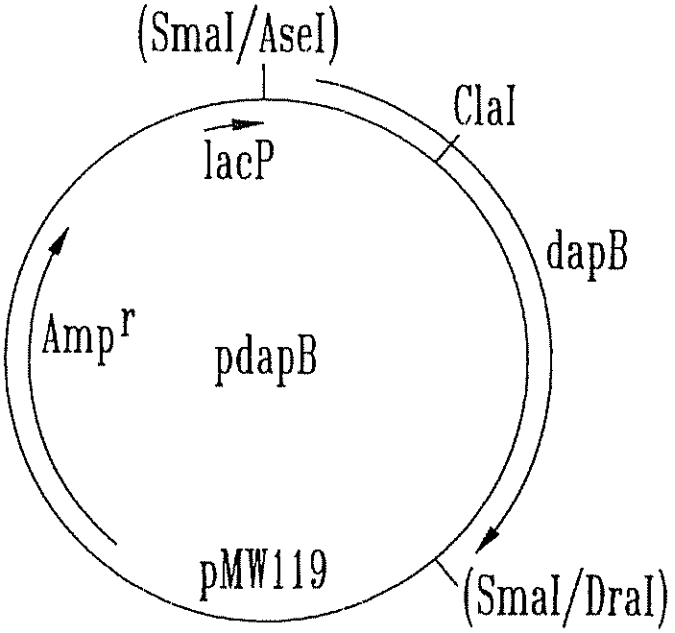
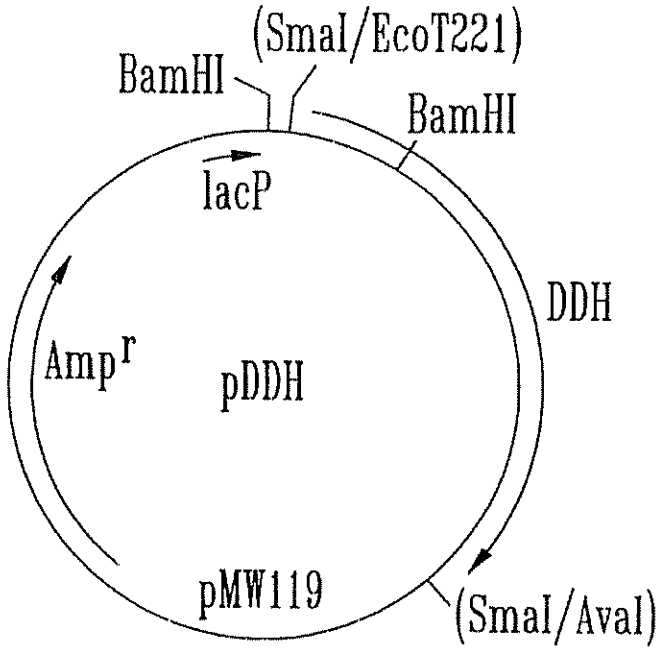

(SmaI/AseI)
ClaI
lacP
dapB
Amp^r
pdapB
pMW119
(SmaI/DraI)

FIG. 15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BamHI
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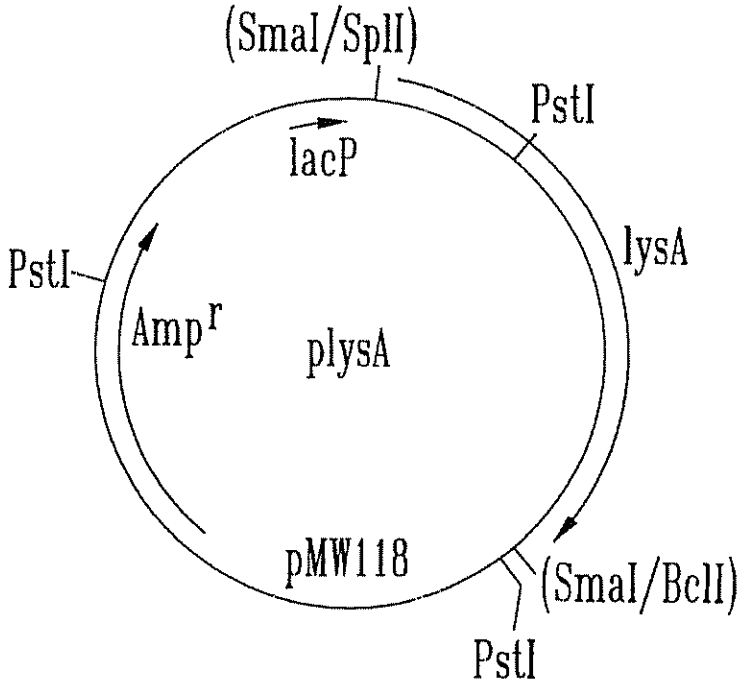
DDH
Amp^r
pDDH
pMW119
(SmaI/AvaI)

FIG. 16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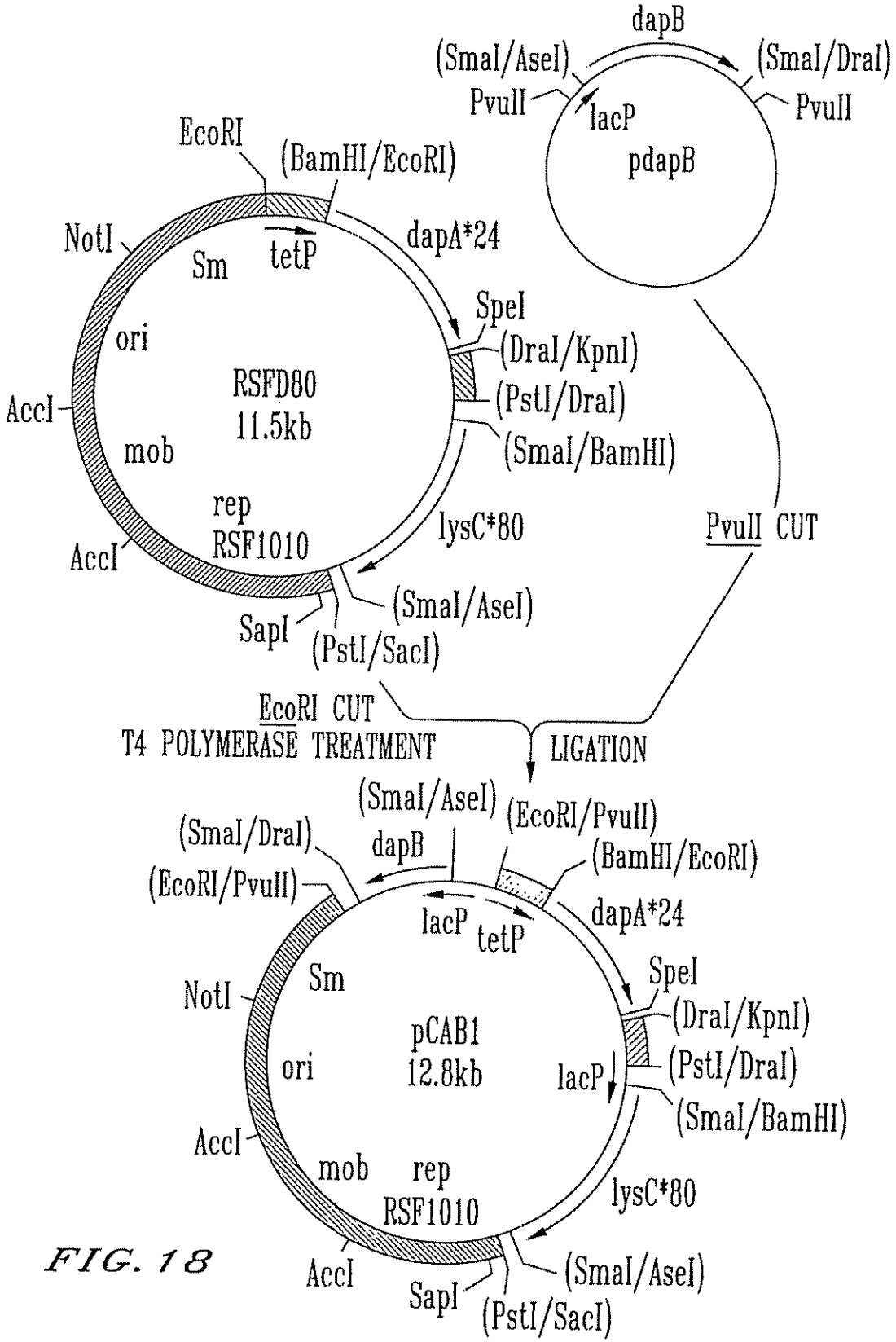
lysA
PstI
Amp^r
plysA
pMW118
(SmaI/BclI)
PstI

FIG. 17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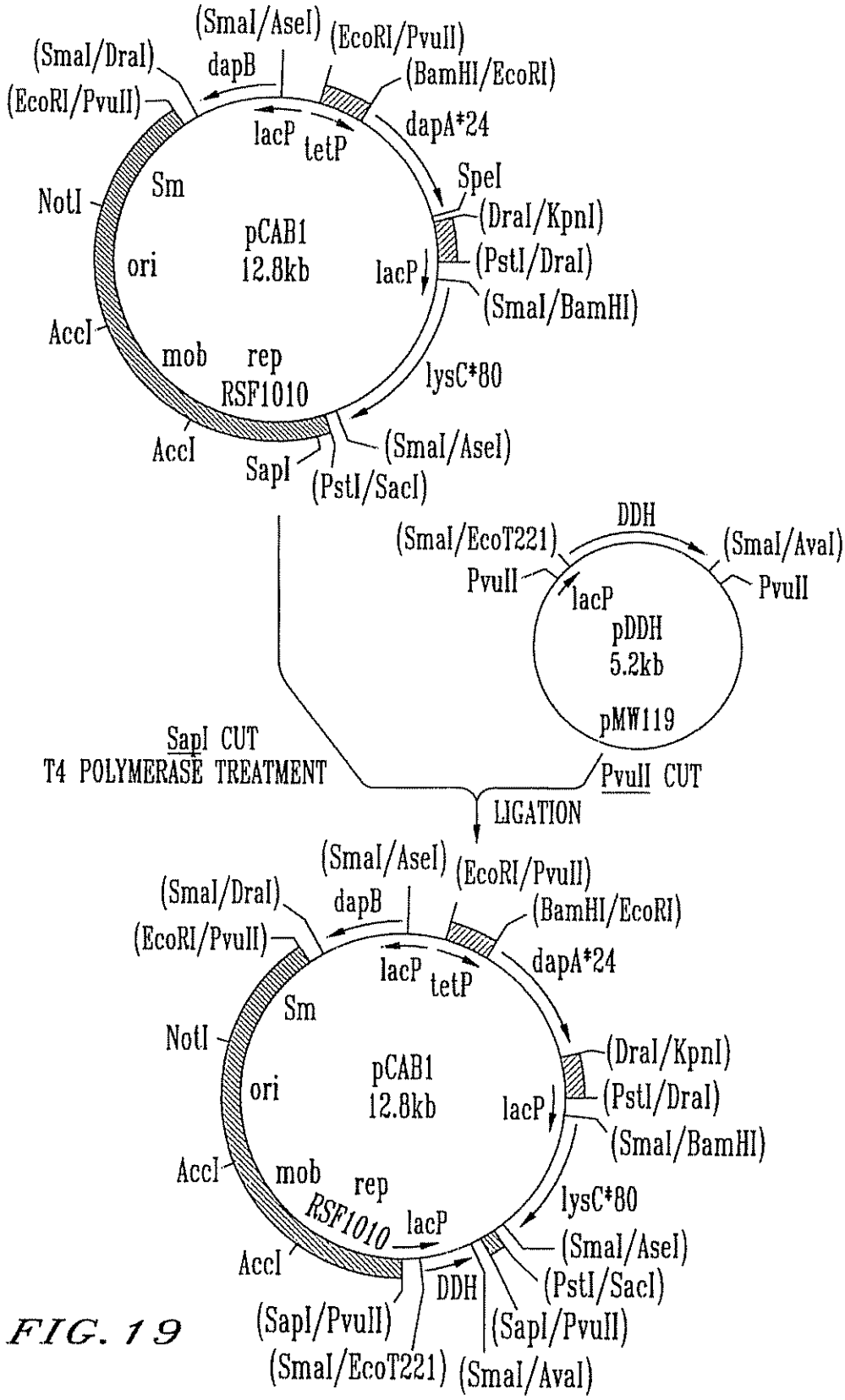
(SmaI/DraI)
PvuII
lacP
PvuII
pdapB
EcoRI
(BamHI/EcoRI)
NotI
Sm
tetP
dapA*24
SpeI
ori
(DraI/KpnI)
RSFD80
11.5kb
AccI
(PstI/DraI)
mob
(SmaI/BamHI)
rep
RSF1010
lysC*80
PvuII CUT
AccI
(SmaI/AseI)
SapI
(PstI/SacI)
EcoRI CUT
T4 POLYMERASE TREATMENT
LIGATION
(SmaI/AseI)
(EcoRI/PvuII)
(SmaI/DraI)
dapB
(BamHI/EcoRI)
(EcoRI/PvuII)
lacP
tetP
dapA*24
Sm
SpeI
NotI
(DraI/KpnI)
pCAB1
12.8kb
ori
lacP
(PstI/DraI)
(SmaI/BamHI)
AccI
mob
rep
RSF1010
lysC*80
AccI
(SmaI/AseI)
SapI
(PstI/SacI)

FIG. 18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(SmaI/DraI)
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(BamHI/EcoRI)
lacP
tetP
dapA*24
SpeI
Sm
NotI
pCAB1
12.8kb
(DraI/KpnI)
ori
lacP
(PstI/DraI)
(SmaI/BamHI)
AccI
mob
rep
RSF1010
lysC*80
AccI
SapI
(SmaI/AseI)
(PstI/SacI)
DDH
(SmaI/EcoT221)
(SmaI/AvaI)
PvuII
PvuII
lacP
pDDH
5.2kb
pMW119
SapI CUT
T4 POLYMERASE TREATMENT
PvuII CUT
LIGATION
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(EcoRI/PvuII)
(SmaI/DraI)
dapB
(BamHI/EcoRI)
(EcoRI/PvuII)
lacP
tetP
dapA*24
Sm
NotI
(DraI/KpnI)
pCAB1
12.8kb
ori
lacP
(PstI/DraI)
(SmaI/BamHI)
AccI
mob
rep
RSF1010
lysC*80
lacP
(SmaI/AseI)
AccI
DDH
(PstI/SacI)
(SapI/PvuII)
(SapI/PvuII)
(SmaI/EcoT221)
(SmaI/AvaI)

FIG. 19

FIG. 20

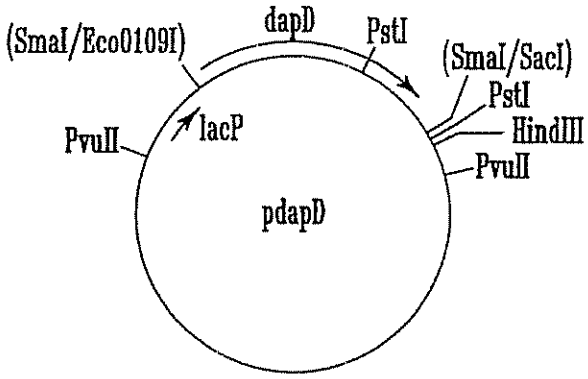


FIG. 21

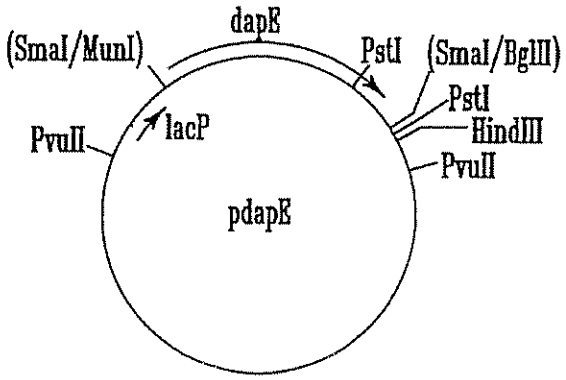


FIG. 22

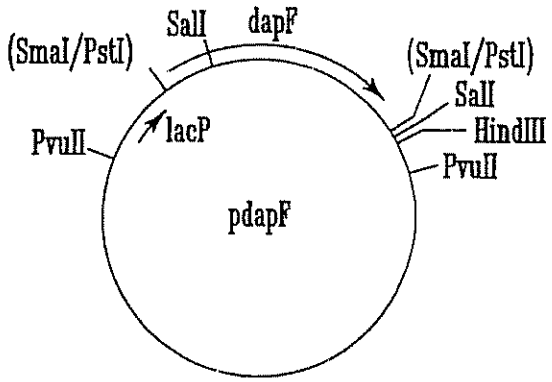
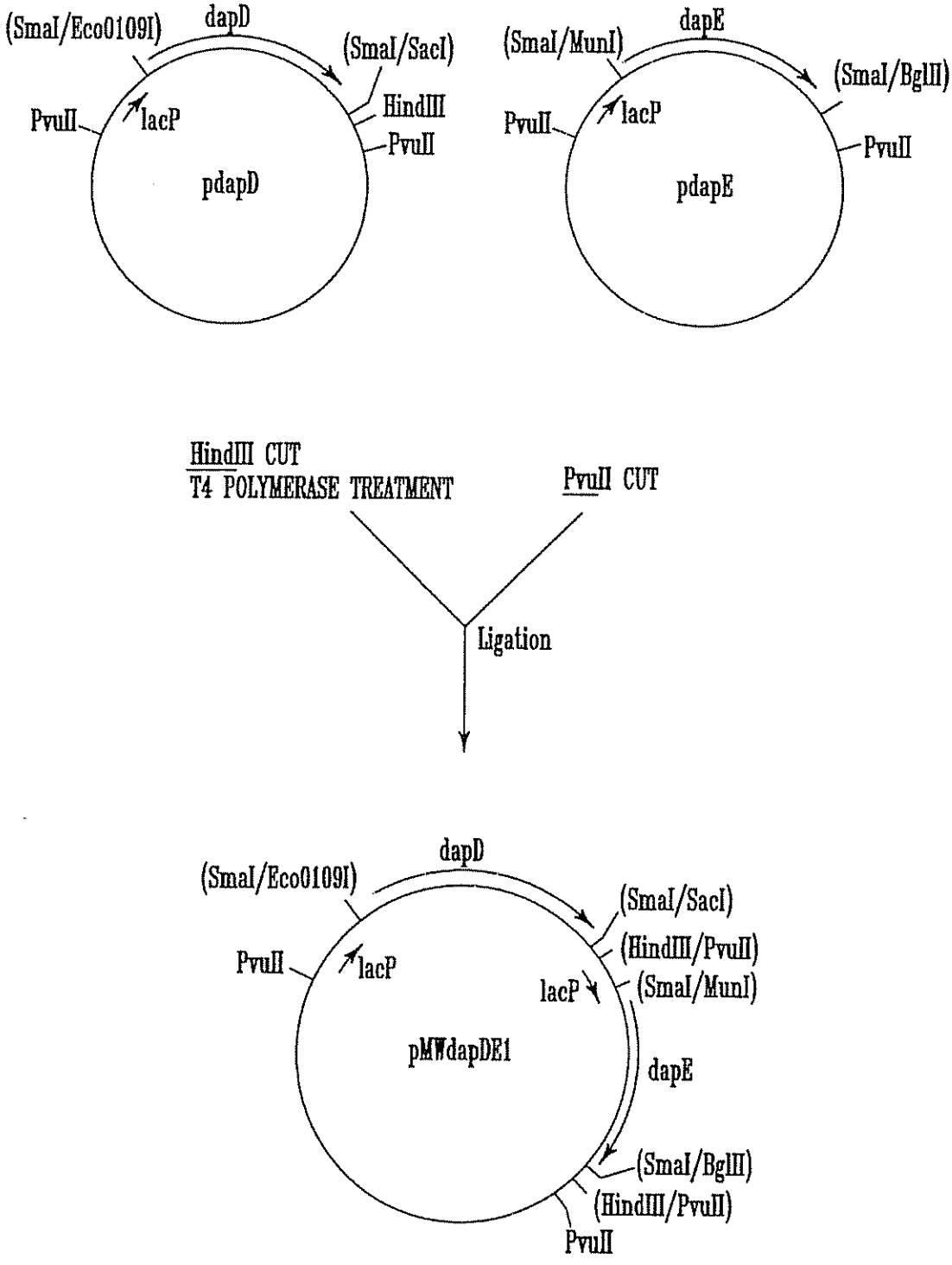


FIG. 23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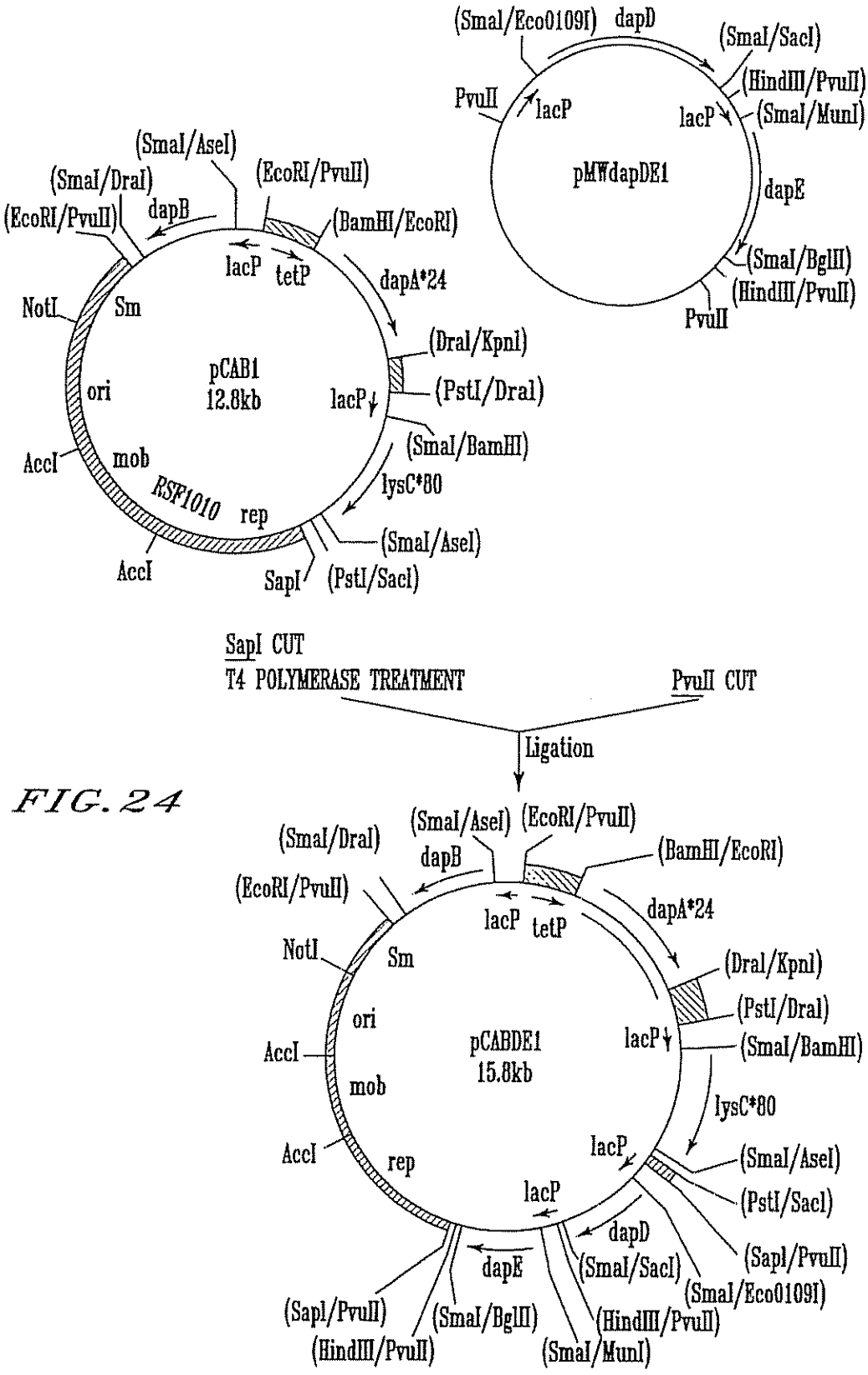
(SmaI/SacI)
HindIII
PvuII
lacP
PvuII
pdapD
(SmaI/MunI)
dapE
(SmaI/BglII)
PvuII
lacP
PvuII
pdapE
HindIII CUT
T4 POLYMERASE TREATMENT
PvuII CUT
Ligation
(SmaI/Eco0109I)
dapD
(SmaI/SacI)
(HindIII/PvuII)
PvuII
lacP
lacP
(SmaI/MunI)
pMWdapDE1
dapE
(SmaI/BglII)
(HindIII/PvuII)
PvuII


(SmaI/Eco0109I)
dapD
(SmaI/SacI)
(HindIII/PvuII)
PvuII
lacP
lacP
(SmaI/MunI)
pMWdapDE1
dapE
(SmaI/BglII)
(HindIII/PvuII)
PvuII
(SmaI/AseI)
(SmaI/DraI)
(EcoRI/PvuII)
dapB
(EcoRI/PvuII)
(BamHI/EcoRI)
lacP
tetP
dapA*24
NotI
Sm
(DraI/KpnI)
pCAB1
12.8kb
ori
(PstI/DraI)
lacP
(SmaI/BamHI)
AccI
mob
lysC*80
RSF1010
rep
(SmaI/AseI)
AccI
SapI
(PstI/SacI)
SapI CUT
T4 POLYMERASE TREATMENT
PvuII CUT
Ligation
(SmaI/AseI)
(EcoRI/PvuII)
(SmaI/DraI)
dapB
(BamHI/EcoRI)
(EcoRI/PvuII)
lacP
tetP
dapA*24
NotI
Sm
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ori
(PstI/DraI)
AccI
pCABDE1
15.8kb
lacP
(SmaI/BamHI)
mob
lysC*80
AccI
rep
lacP
(SmaI/AseI)
lacP
(PstI/SacI)
dapD
(SapI/PvuII)
dapE
(SmaI/SacI)
(SmaI/Eco0109I)
(SapI/PvuII)
(SmaI/BglII)
(HindIII/PvuII)
(HindIII/PvuII)
(SmaI/MunI)

FIG. 24

METHOD OF PRODUCING L-LYSINE BY FERMENTATION

TECHNICAL FIELD

The present invention relates to microbial industry, and in particular relates to a method of producing L-lysine by fermentation, DNA's and microorganisms to be used for this production method.

BACKGROUND ART

In the prior art, when L-lysine is produced by a fermentative method, a microbial strain separated from the natural environment or an artificial mutant strain obtained from such a microbial strain is used in order to improve the productivity A large number of artificial mutant strains producing L-lysine are known. Most of them are S-2-aminoethylcysteine (AEC) resistant mutant strains, and belong to the genus of Brevibacterium, Corynebacterium, Bacillus or Escherichia. Further, various techniques have been disclosed for increasing amino acid production, for example, by employing a transformant using recombinant DNA (U.S. Pat. No. 4,278,765)

With respect to those belonging to the genus Escherichia, for example, Japanese Patent Application Laid-open No. 56-18596, U.S. Pat. No. 4,346,170, and *Applied Microbiology and Biotechnoloay*, 15, 227–231 (1982) describe methods of producing L-lysine using a bacterial strain in which dihydrodipicolinate synthase (hereinafter sometimes abbreviated as "DDPS") is enhanced. However, DDPS used in these cases is a wild type, which suffers feedback inhibition by L-lysine. Thus sufficiently satisfactory L-lysine productivity has not been obtained. Incidentally, *Applied Microbiology and Biotechnology*, 15, 227–231 (1982) mentioned above is describes an L-lysine production of 3 g/l of L-lysine hydrochloride from 75 g/l of glucose, wherein a consumption coefficient (number of g of L-lysine produced from 1 g of sugar, or percentage thereof) is calculated to be 0.04, or 4%.

On the other hand, Korean Patent Publication No. 92–8382 describes a method of producing L-lysine using a bacterium belonging to Escherichia to which DDPS originating from a bacterium belonging to the genus Corynebacterium, which is known not to suffer feedback inhibition by L-lysine (consumption coefficient: 17%), is introduced. However, the upper limit temperature for growth of bacteria belonging to the genus Corynebacterium is lower than the upper limit temperature for growth of bacteria belonging to the genus Escherichia by about 10 degrees. Thus it seems that cultivation should be performed at a lowered cultivation temperature if DNA coding for DDPS originating from a bacterium belonging to the genus Corynebacterium is introduced into a bacterium belonging to the genus Escherichia in order to utilize it for L-lysine production Therefore, it is anticipated that it is difficult to exhibit advantages possessed by the bacterium belonging to the genus Escherichia that the growth temperature is high, the growth speed is fast, and the L-lysine-producing speed is also fast Generally, when a gene originating from a heterologous organism is expressed, there are occasionally caused decomposition of an expression product by protease and formation of an insoluble inclusion body, in which more difficulties are anticipated as compared with a case of expression of a homologous gene. Further, when DNA coding for DDPS originating from a bacterium belonging to the genus Corynebacterium is introduced into a bacterium belonging to the genus Escherichia to industrially produce L-lysine, more strict regulation is obliged as compared with a case of use of a recombinant to which a homologous gene is introduced, in accordance with the recombinant DNA guideline.

By the way, the dihydrodipicolinate synthase (DDPS) is an enzyme for dehydrating and condensing aspartosemialdehyde and pyruvic acid to synthesize dihydrodipicolinic acid This reaction is located at an entrance into a branch to proceed to an L-lysine biosynthesis system in biosynthesis of amino acids of the aspartic acid family. This enzyme is known to be in charge of an important regulatory site as aspartokinase is in bacteria belonging to the genus Escherichia.

DDPS is encoded by a gene called dapA in *E. coli* (*Escherichia coli*) The dapA has been cloned, and its nucleotide sequence has been also determined (Richaud, F et al., *J Bacteriol*, 297 (1986))

On the other hand, aspartokinase (hereinafter sometimes abbreviated as "AK") is an enzyme for catalyzing a reaction to convert aspartic acid into β-phosphoaspartic acid, which serves as a main regulatory enzyme in a biosynthesis system of amino acids of the aspartic acid family. AK of *E. coli* has three types (AKI, AKII, AKIII), two of which are complex enzymes with homoserine dehydrogenase (hereinafter sometimes abbreviated as "HD") One of the complex enzymes is AKI-HDI encoded by a thrA gene, and the other is AKII-HDII encoded by a metLM gene. AKI is subjected to concerted suppression by threonine and isoleucine and inhibited by threonine, while AKII is suppressed by methionine.

On the contrary, it is known that only AKIII is a simple function enzyme, which is a product of a gene designated as lysC, and is subjected to suppression and feedback inhibition by L-lysine. The ratio of their intracellular activities is AKI:AKII:AKIII=about 5:1:4.

As described above, DDPS originating from bacteria belonging to the genus Corynebacterium is not subjected to feedback inhibition by L-lysine. However, when it is introduced into a bacterium belonging to the genus Escherichia to utilize it for L-lysine production, a problem arises in the cultivation temperature. It is expected that L-lysine can be efficiently produced by fermentation by using a bacterium belonging to the genus Escherichia if a mutant enzyme of DDPS or AKIII originating from a bacterium belonging to the genus Escherichia, which is not subjected to feedback inhibition by L-lysine, can be obtained However, there is no preceding literature which describes such a mutant enzyme of DDPS, and although there is one report on a mutant enzyme of AKIII (Boy, E., et al., J. Bacteriol., 112, 84 (1972)) no example has been known which suggests that such a mutant enzyme may improve productivity of L-lysine.

DISCLOSURE OF THE INVENTION

The present invention has been made taking the aforementioned viewpoints into consideration, an object of which is to obtain DDPS and AKIII originating from bacteria belonging to the genus Escherichia with sufficiently desensitized feedback inhibition by L-lysine, and provide a method of producing L-lysine by fermentation which is more improved than those in the prior art.

As a result of diligent and repeated investigation in order to achieve the object described above, the present inventors have succeeded in obtaining DNA coding for DDPS originating from a bacterium belonging to the genus Escherichia in which feedback inhibition by L-lysine is sufficiently

desensitized. The DNA coding for DDPS originating from *E. coli* in which feedback inhibition by L-lysine is sufficiently desensitized is sometimes referred to herein as mutant dapA or dapA*.

The inventors have further created a bacterium belonging to the genus Escherichia harboring mutant dapA and aspartokinase which is desensitized feedback inhibition by L-lysine. The DNA coding for aspartokinase originating from *E. coli* in which feedback inhibition by L-lysine is sufficiently desensitized is sometimes referred to herein as mutant lysC or lysC*.

The inventors have further created a bacterium belonging to the genus Escherichia harboring mutant dapA and mutant lysC. And it has been found that a considerable amount of L-lysine can be produced and accumulated in a culture by cultivating the aforementioned bacterium belonging to the genus Escherichia in a preferred medium.

The inventors have still further found that the productivity of L-lysine can be further improved by enhancing other genes in the L-lysine biosynthesis system of a bacterium belonging to the genus Escherichia harboring the mutant dapA and the mutant lysC.

Namely, the present invention lies in a DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus Escherichia having mutation to desensitize feedback inhibition by L-lysine. The mutation to desensitize feedback inhibition by L-lysine is exemplified by mutation selected from the group consisting of mutation to replace a 81st alanine residue with a valine residue, mutation to replace a 118th histidine residue with a tyrosine residue, and mutation to replace the 81st alanine residue with the valine residue and replace the 118th histidine residue with the tyrosine residue, as counted from the N-terminal in an amino acid sequence of dihydrodipicolinate synthase defined in SEQ ID NO:4 in Sequence Listing.

The present invention further lies in a bacterium belonging to the genus Escherichia transformed by introducing, into its cells, a DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus Escherichia having mutation to desensitize feedback inhibition by L-lysine. The mutation to desensitize feedback inhibition by L-lysine is exemplified by mutation to replace a 81st alanine residue with a valine residue, mutation to replace a 118th histidine residue with a tyrosine residue, and mutation to replace the 81st alanine residue with the valine residue and replace the 118th histidine residue with the tyrosine residue, as counted from the N-terminal in an amino acid sequence of dihydrodipicolinate synthase defined in SEQ ID NO:4 in Sequence Listing.

The present invention further lies in the aforementioned bacterium belonging to the genus Escherichia harboring an aspartokinase which is also desensitized feedback inhibition by L-lysine. A method to allow the bacterium belonging to the genus Escherichia to harbor the aspartokinase which is desensitized feedback inhibition by L-lysine is exemplified by a method for introducing, into its cells, a DNA coding for an aspartokinase III originating from a bacterium belonging to the genus Escherichia having mutation to desensitize feedback inhibition by L-lysine.

The mutation of the aspartokinase III to desensitize feedback inhibition by L-lysine is exemplified by mutation to replace a 323rd glycine residue with an aspartic acid residue, mutation to replace the 323rd glycine residue with the aspartic acid residue and replace a 408th glycine residue with an aspartic acid residue, mutation to replace a 34th arginine residue with a cysteine residue and replace the 323rd glycine residue with the aspartic acid residue, mutation to replace a 325th leucine residue with a phenylalanine residue, mutation to replace a 318th methionine residue with an isoleucine residue, mutation to replace the 318th methionine residue with the isoleucine residue and replace a 349th valine residue with a methionine residue, mutation to replace a 345th serine residue with a leucine residue, mutation to replace a 347th valine residue with a methionine residue, mutation to replace a 352nd threonine residue with an isoleucine residue, mutation to replace the 352nd threonine residue with the isoleucine residue and replace a 369th serine residue with a phenylalanine residue, mutation to replace a 164th glutamic acid residue with a lysine residue, and mutation to replace a 417th methionine residue with an isoleucine residue and replace a 419th cysteine residue with a tyrosine residue, as counted from the N-terminal in an amino acid sequence of aspartokinase III defined in SEQ ID NO:8 in Sequence Listing.

The DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus Escherichia having mutation to desensitize feedback inhibition by L-lysine, and the DNA coding for an aspartokinase III having mutation to desensitize feedback inhibition by L-lysine may be harbored on a chromosome of a bacterium belonging to the genus Escherichia respectively, or may be harbored in cells on an identical plasmid or separate plasmids. Further, it is also acceptable that one of the respective DNA's is harbored on a chromosome, and the other DNA is harbored on a plasmid.

The present invention still further lies in the aforementioned bacterium belonging to the genus Escherichia wherein a dihydrodipicolinate reductase gene is enhanced. The enhancement of the dihydrodipicolinate reductase gene can be achieved by transformation with a recombinant DNA constructed by ligating the dihydrodipicolinate reductase gene with a vector autonomously replicable in cells of bacteria belonging to the genus Escherichia.

The present invention further lies in the aforementioned bacterium belonging to the genus Escherichia wherein an enhanced diaminopimelate dehydrogenase gene originating from coryneform bacteria such as *Brevibacterium lactofermentum* is introduced. The introduction of the enhanced diaminopimelate dehydrogenase gene originating from coryneform bacteria can be achieved by transformation with a recombinant DNA constructed by ligating the gene with a vector autonomously replicable in cells of bacteria belonging to the genus Escherichia. As coryneform bacteria, there may be exemplified wild type strains producing glutamic acid, and mutant strains thereof producing other amino acids, which belong to the genus Corynebacterium or the genus Brevibacterium. More concretely, *Brevibacterium flavum, Brevibacterium divaricatum, Corynebacterium glutamicum* and *Corynebacterium lilium* as well as *Brevibacterium lactofermentum* are exemplified as coryneform bacteria used for the present invention.

The present invention further lies in the bacterium belonging to the genus Escherichia wherein a tetrahydrodipicotinate succinylase gene and a succinyldiaminopmelate deacylase gene are enhanced instead of the aforementioned diaminodipimelate dehydrogenase gene. The enhancement of these genes can be achieved by transformation with a single recombinant DNA or two recombinant DNA's constructed by ligating these genes with an identical vector or different vectors autonomously replicable in cells of bacteria belonging to the genus Escherichia.

The present invention further provides a method of producing L-lysine comprising the steps of cultivating any of

the bacteria belonging to the genus Escherichia described above in an appropriate medium, producing and accumulating L-lysine in a culture thereof, and collecting L-lysine from the culture.

In this specification, DNA coding for DDPS or AKIII, or DNA containing a promoter in addition thereto is sometimes referred to as "DDPS gene" or "AKIII gene". Further, the mutant enzyme which is desensitized feedback inhibition by L-lysine, and DNA coding for it or DNA containing a promoter in addition to it are sometimes simply refereed to as "mutant enzyme" and "mutant gene", respectively. Further, the phrase "feedback inhibition by L-lysine is desensitized" means that substantial desensitization of inhibition is sufficient, and complete desensitization is not necessary.

The present invention will be explained in detail below.

<1> DNA Coding for Mutant Dihydrodipicolinate Synthase (DDPS) of the Present Invention

The DNA coding for the mutant DDPS of the present invention has mutation to desensitize feedback inhibition by L-lysine of DDPS encoded in DNA coding for the wild type DDPS. DDPS is exemplified by those originating from bacteria belonging to the genus Escherichia, especially DDPS originating from *E. coli* The mutation of DDPS to desensitize feedback inhibition by L-lysine is exemplified by:

(1) mutation to replace a 81st alanine residue with a valine residue;

(2) mutation to replace a 118th histidine residue with a tyrosine residue; and

(3) mutation to replace the 81st alanine residue with the valine residue and replace the 118th histidine residue with the tyrosine residue; as counted from the N-terminal of DDPS in an amino acid sequence of DDPS defined in SEQ ID NO:4 in Sequence Listing.

The DNA coding for the wild type DDPS is not especially limited provided that it codes for DDPS originating from a bacterium belonging to the genus Escherichia, which is concretely exemplified by DNA coding for an amino acid sequence defined in SEQ ID NO:4, and is further concretely exemplified by a sequence represented by base numbers 272–1147 in a base sequence defined in SEQ ID NO:3. In these sequences, those having the mutation in nucleotide sequence to cause the replacement of amino acid residues described above are the DNA coding for the mutant DDPS of the present invention. Any codon corresponding to the replaced amino acid residue is available especially irrelevantly to its kind, provided that it codes for the identical amino acid residue. Further, it is postulated that possessed DDPS is slightly different in sequence depending on difference in bacterial species and bacterial strain, however, those having replacement, deletion or insertion of amino acid residue(s) at position(s) irrelevant to enzyme activity are also included in the mutant DDPS gene of the present invention.

A method for obtaining such a mutant gene is as follows. At first, a DNA containing a wild type DDPS gene or DDPS gene having another mutation is subjected to an in vitro mutation treatment, and a DNA after the mutation treatment is ligated with a vector DNA adapted to a host to obtain a recombinant DNA. The recombinant DNA is introduced into a host microorganism to obtain transformants. When one which expresses a mutant DDPS is selected among the aforementioned transformants, such a transformant harbors a mutant gene. Alternatively, a DNA containing a wild type DDPS gene or DDPS gene having another mutation may be ligated with a vector DNA adapted to a host to obtain a recombinant DNA. The recombinant DNA is thereafter subjected to an in vitro mutation treatment, and a recombinant DNA after the mutation treatment is introduced into a host microorganism to obtain transformants. When one which expresses a mutant DDPS is selected among the aforementioned transformants, such a transformant also harbors a mutant gene.

It is also acceptable that a microorganism which, produces a wild type enzyme is subjected to a mutation treatment to create a mutant strain which produces a mutant enzyme, and then a mutant gene is obtained from the mutant strain. Alternatively, a transformant to which a recombinant DNA ligated with a wild type gene is introduced may be subjected to a mutation treatment to create a mutant strain which produces a mutant enzyme. When a recombinant DNA is thereafter recovered from the mutant strain, a mutant gene is created on the aforementioned DNA.

The agent for performing the in vitro mutation treatment of DNA is exemplified by hydroxylamine and the like. Hydroxylamine is a chemical mutation treatment agent which causes mutation from cytosine to thymine by changing cytosine to N^4-hydroxycytosine. Alternatively, when a microorganism itself is subjected to a mutation treatment, the treatment is performed by using ultraviolet light irradiation, or a mutating agent usually used for artificial mutation such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or nitrous acid.

No problem occurs when any one is used as a donor microorganism for DNA containing the wild type DDPS gene or DDPS gene having another mutation described above, provided that it is a microorganism belonging to the genus Escherichia. Concretely, it is possible to utilize those described in a book written by Neidhardt et al. (Neidhardt, F. C. et al., *Escherichia coli* and *Salmonella Typhimurium*, American Society for Microbiology, Washington D. C., 1208, table 1). For example, an *E. coli* JM109 strain and an MC1061 strain are exemplified. When a wild strain is used as a donor microorganism for DNA containing a DDPS gene, a DNA containing a wild type DDPS gene can be obtained.

(1) Preparation of Wild Type DDPS Gene

An example of preparation of DNA containing a DDPS gene will be described below. At first, *E. coli* having wild type dapA, for example, MC1061 strain, is cultivated to obtain a culture. When the microorganism described above is cultivated, cultivation may be performed in accordance with an ordinary solid culture method, however, cultivation is preferably performed by adopting a liquid culture method considering efficiency during collection of the bacterium. A medium may be used in which one or more nitrogen sources such as yeast extract, peptone, meat extract, corn steep liquor and exudate of soybean or wheat are added with one or more inorganic salts such as potassium dihydrogenphosphate, dipotassium hydrogenphosphate, magnesium sulfate, sodium chloride, magnesium chloride, ferric chloride, ferric sulfate or manganese sulfate, and further optionally and adequately added with sugar materials, vitamins and the like. It is appropriate that the initial pH of the medium is adjusted to 6–8. The cultivation is performed for 4–24 hours at 30–42° C., preferably at about 37° C. by means of deep culture with aeration and agitation, shaking culture or stationary culture or the like.

The culture thus obtained is centrifuged, for example, at 3,000 r.p.m. for 5 minutes to obtain a cell pellet of *E. coli* MC1061 strain. Chromosomal DNA can be obtained from the cell pellet by means of, for example, a method of Saito

and Miura (*Biochem Biophys Acta*, 72, 619 (1963)), or a method of K. S. Kirby (*Biochem. J.*, 64, 405 (1956)).

In order to isolate the DDPS gene from the chromosomal DNA thus obtained, a chromosomal DNA library is prepared. At first, the chromosomal DNA is partially digested with a suitable restriction enzyme to obtain a mixture of various fragments. A wide variety of restriction enzymes can be used if the degree of cutting is controlled by the cutting reaction time and the like. For example, Sau3AI is allowed to react on the chromosomal DNA at a temperature not less than 30° C., preferably at 37° C. at an enzyme concentration of 1–10 units/ml for various periods of time (1 minute to 2 hours) to digest it.

Next, obtained DNA fragments are ligated with a vector DNA autonomously replicable in cells of bacteria belonging to the genus Escherichia to prepare recombinant DNA. Concretely, a restriction enzyme, which generates the terminal nucleotide sequence complement to that generated by the restriction enzyme Sau3AI used to cut the chromosomal DNA, for example, BmHI, is allowed to act on the vector DNA under a condition of a temperature not less than 30° C. and an enzyme concentration of 1–100 units/ml for not less than 1 hour, preferably for 1–3 hours to completely digest it, and cut and cleave it. Next, the chromosomal DNA fragment mixture obtained as described above is mixed with the cleaved and cut vector DNA, on which DNA ligase, preferably T4 DNA ligase is allowed to act under a condition of a temperature of 4–16° C. at an enzyme concentration of 1–100 units/ml for not less than 1 hour, preferably for 6–24 hours to obtain recombinant DNA.

The obtained recombinant DNA is used to transform a microorganism belonging to the genus Escherichia, for example, a DDPS deficient mutant strain such as an *Escherichia coli* K-12 strain, preferably a JE7627 strain (ponB704, dacB12, pfv$^+$, tonA2, dapA, lysA, str, malA38, metB1, ilvH611, leuA371, proA3, lac-3, tsx-76) to prepare a chromosomal DNA library. The transformation can be performed, for example, by a method of D. M. Morrison (*Methods in Enzymology* 68, 326 (1979)) or a method in which recipient bacterial cells are treated with calcium chloride to increase permeability of DNA (Mandel, M. and Higa, A., *J. Mol. Biol.*, 53, 159 (1970)). The JE7627 strain is available from National Institute of Genetics (Mishima-shi, Shizuoka-ken, Japan).

A bacterial strain having recombinant DNA of the DDPS gene is obtained from strains having increased DDPS activity or strains in which auxotrophy resulting from deficiency in DDPS gene is complemented, among the obtained chromosomal DNA library. For example, a DDPS deficient mutant strain requires diaminopimelic acid. Thus when the DDPS deficient mutant strain is used as a host, a DNA fragment containing the DDPS gene can be obtained by isolating a bacterial strain which becomes capable of growing on a medium containing no diaminopimelic acid, and recovering recombinant DNA from the bacterial strain.

Confirmation of the fact whether or not a candidate strain having recombinant DNA containing a DDPS gene actually harbors recombinant DNA in which the DDPS gene is cloned can be achieved by preparing a cellular extract from the candidate strain, and preparing a crude enzyme solution therefrom to confirm whether or not the DDPS activity has been increased. A procedure to measure the enzyme activity of DDPS can be performed by a method of Yugari et al. (Yugari, Y. and Gilvarg, C., *J. Biol. Chem.*, 240, 4710 (1962)).

Recombinant DNA in which DNA containing the DDPS gene is inserted into the vector DNA can be isolated from the bacterial strain described above by means of, for example, a method of P. Guerry et al. (*J. Bacteriol.*, 116, 1064 (1973)) or a method of D. B. Clewell (*J. Bacteriol.*, 110, 667 (1972)).

Preparation of the wild type DDPS gene can be also performed by preparing chromosomal DNA from a strain having a DDPS gene on chromosome by means of a method of Saito and Miura or the like, and amplifying the DDPS gene by means of a polymerase chain reaction (PCR) method (see White, T. J. et al.; *Trends Genet.*, 5, 185 (1989)). DNA primers to be used for the amplification reaction are those complemental to both 3'-terminals of a double stranded DNA containing an entire region or a partial region of the DDPS gene. When only a partial region of the DDPS gene is amplified, it is necessary to use such DNA fragments as primers to perform screening of a DNA fragment containing the entire region from a chromosomal DNA library. When the entire region of the DDPS gene is amplified, a PCR reaction solution including DNA fragments containing the amplified DDPS gene is subjected to agarose gel electrophoresis, and then an aimed DNA fragment is extracted. Thus a DNA fragment containing the DDPS gene can be recovered.

The DNA primers may be adequately prepared on the basis of, for example, a sequence known in *E. coli* (Richaud, F. et al., *J. Bacteriol.*, 297 (1986)). Concretely, primers which can amplify a region comprising 1150 bases coding for the DDPS gene are preferable, and two species of primers defined in SEQ ID NO:1 and NO:2 are suitable. Synthesis of the primers can be performed by an ordinary method such as a phosphoamidite method (see *Tetrahedron Letters*, 22, 1859 (1981)) by using a commercially available DNA synthesizer (for example, DNA Synthesizer Model 380B produced by Applied Biosystems Inc.). Further, the PCR can be performed by using a commercially available PCR apparatus (for example, DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo Co., Ltd.), using Tag DNA polymerase (supplied by Takara Shuzo Co., Ltd.) in accordance with a method designated by the supplier.

With respect to the DDPS gene amplified by the PCR method, operations such as introduction of mutation into the DDPS gene become easy, when it is ligated with a vector DNA autonomously replicable in cells of bacteria belonging to the genus Escherichia, and introduced into cells of bacteria belonging to the genus Escherichia. The vector DNA to be used, the transformation method, and the confirmation method for the presence of the DDPS gene are the same as those in the aforementioned procedure.

(2) Introduction of Mutation Into DDPS Gene

The method for carrying out mutation such as replacement, insertion and deletion of amino acid residues is exemplified by a recombinant PCR method (Higuchi, R., 61, in *PCR Technology* (Erlich, H. A. Eds., Stockton press (1989))), and a site specific mutagenesis method (Kramer, W. and Frits, H. J., *Meth. in Enzymol.*, 154, 350 (1987); Kunkel T. A. et al., *Meth. in Enzymol.*, 154, 367 (1987)). Aimed mutation can be caused at an aimed site by using these methods.

Further, according to chemical synthesis of an aimed gene, it is possible to introduce mutation or random mutation into an aimed site.

Further, a method is available in which the DDPS gene on chromosome or plasmid is directly treated with hydroxylamine (Hashimoto, T. and Sekiguchi, M. *J. Bacteriol.*, 159, 1039 (1984)). Alternatively, it is acceptable to use a method in which a bacterium belonging to the genus Escherichia having the DDPS gene is irradiated by ultraviolet light, or a

method based on a treatment with a chemical agent such as N-methyl-N'-nitrosoguanidine or nitrous acid. According to these methods, mutation can be introduced randomly.

With respect to a selection method for the mutant gene, recombinant DNA comprising a DNA fragment containing the DDPS gene and vector DNA is at first directly subjected to a mutation treatment with hydroxylamine or the like, which is used to transform, for example, an *E. coli* W3110 strain. Next, transformed strains are cultivated on a minimal medium such as M9 containing S-2-aminoethylcysteine (AEC) as an analog of L-lysine. Strains harboring recombinant DNA containing the wild type DDPS gene cannot synthesize L-lysine and diaminopimelic acid (DAP) and are suppressed in growth because DDPS expressed from the recombinant DNA is inhibited by AEC. On the contrary, a strain harboring recombinant DNA containing the DDPS gene in which inhibition by L-lysine is desensitized has a mutant enzyme encoded by the DDPS gene in the aforementioned recombinant DNA which is not inhibited by AEC. Thus it should be capable of growth on the minimal medium in which AEC is added. This phenomenon can be utilized to select a strain which is resistant in growth to AEC as an analog of L-lysine, that is a strain harboring recombinant DNA containing a mutant DDPS gene in which inhibition is desensitized.

The mutant gene thus obtained may be introduced as a recombinant DNA into a suitable host microorganism, and expressed. Thus a microorganism can be obtained which harbors DDPS being desensitized feedback inhibition. The host is preferably a microorganism belonging to the genus Escherichia, for which *E. coli* is exemplified.

Alternatively, a mutant DDPS gene fragment may be taken out from the recombinant DNA, and inserted into another vector to make use. The vector DNA which can be used in the present invention is preferably plasmid vector DNA, for which there are exemplified pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219 and pMW218. Besides, vectors of phage DNA can be also utilized.

Further, in order to express the mutant DDPS gene efficiently, another promoter which works in microorganisms such as lac, trp and PL may be ligated upstream from a DNA sequence coding for the mutant DDPS, or a promoter contained in the DDPS gene may be used as it is, or after amplifying the promoter.

In addition, as described above, the mutant gene may be inserted into an autonomously replicable vector DNA, which is inserted into a host, and allowed to be harbored by the host as extrachromosomal DNA such as a plasmid. Alternatively, the mutant gene may be integrated into chromosome of a host microorganism by a method using transduction, transposon (Berg, D. E. and Berg, C. M., *Bio/Technol.*, 1, 417 (1983)), Mu phage (Japanese Patent Application Laid-open No. 2-109985) or homologous recombination (*Experiments in Molecular Genetics*, Cold Spring Harbor Lab. (1972)).

<2> DNA Coding for Mutant Aspartokinase III (AKIII) Used for the Present Invention

The DNA coding for mutant AKIII used for the present invention has mutation to desensitize feedback inhibition of encoded AKIII by L-lysine in DNA coding for wild type AKIII. The mutation to desensitize feedback inhibition of AKIII by L-lysine is exemplified by:

(a) mutation to replace a 323rd glycine residue with an aspartic acid residue;

(b) mutation to replace the 323rd glycine residue with the aspartic acid residue and replace a 408th glycine residue with an aspartic acid residue;

(c) mutation to replace a 34th arginine residue with a cysteine residue and replace the 323rd glycine residue with the aspartic acid residue;

(d) mutation to replace a 325th leucine residue with a phenylalanine residue;

(e) mutation to replace a 318th methionine residue with an isoleucine residue;

(f) mutation to replace the 318th methionine residue with the isoleucine residue and replace a 349th valine residue with a methionine residue;

(g) mutation to replace a 345th serine residue with a leucine residue;

(h) mutation to replace a 347th valine residue with a methionine residue;

(i) mutation to replace a 352nd threonine residue with an isoleucine residue;

(j) mutation to replace the 352nd threonine residue with the isoleucine residue and replace a 369th serine residue with a phenylalanine residue;

(k) mutation to replace a 164th glutamic acid residue with a lysine residue; and

(l) mutation to replace a 417th methionine residue with an isoleucine residue and replace a 419th cysteine residue with a tyrosine residue;

as counted from the N-terminal of AKIII in an amino acid sequence of AKIII defined in SEQ ID NO:8 in Sequence Listing.

The DNA coding for the wild type AKIII is not especially limited, for which DNA coding for AKIII originating from a bacterium belonging to the genus Escherichia such as *E. coli* is exemplified. Concretely, there are exemplified DNA coding for an amino acid sequence defined in SEQ ID NO:8, and a sequence represented by base numbers 584–1930 in a base sequence defined in SEQ ID NO:7. Incidentally, AKIII of *E. coli* is encoded by a lysC gene.

In these sequences, those which have mutation in base sequence to cause replacement of amino acid residues described above are DNA coding for the mutant AKIII of the present invention. Any codon corresponding to the replaced amino acid residue is available especially regardless of its kind, provided that it codes for the identical amino acid residue. Further, there are those in which amino acid sequences of possessed wild type AKIII are slightly different depending on difference in bacterial species and bacterial strains. Those having replacement, deletion or insertion of amino acid residue(s) at position(s) irrelevant to enzyme activity in such a manner are also included in the mutant AKIII gene of the present invention. For example, a base sequence of a wild type lysC gene obtained in Example 2 described below (SEQ ID NO:7) is different from an already published sequence of lysC of an *E. coli* K-12 JC411 strain at 6 sites (Cassan, M., Parsot, C., Cohen, G. N., and Patte, J. C., *J. Biol. Chem.*, 261 1052 (1986)). Encoded amino acid residues are different at 2 sites of them (in lysC of the JC411 strain, a 58th glycine residue is replaced with a cysteine residue, and a 401st glycine residue is replaced with an alanine residue, as counted from the N-terminal in an amino acid sequence of lysC defined in SEQ ID NO:8). It is expected even for lysC having the same sequence as that of lysC of the *E. coli* K-12 JC411 strain that lysC having mutation in which feedback inhibition by L-lysine is desensitized is obtained if any of the aforementioned mutation of (a) to (l) is introduced.

A method for obtaining DNA coding for the mutant AKIII in which feedback inhibition by L-lysine is desensitized is as follows. At first, a DNA containing a wild type AKIII gene

or AKIII gene having another mutation is subjected to an in vitro mutation treatment, and a DNA after the mutation treatment is ligated with a vector DNA adapted to a host to obtain a recombinant DNA. The recombinant DNA is introduced into a host microorganism to obtain transformants. When one which expresses a mutant AKIII is selected among the aforementioned transformants, such a transformant harbors a mutant gene. Alternatively, a DNA containing a wild type AKIII gene or AKIII gene having another mutation may be ligated with a vector DNA adapted to a host to obtain a recombinant DNA. The recombinant DNA is thereafter subjected to an in vitro mutation treatment, and a recombinant DNA after the mutation treatment is introduced into a host microorganism to obtain transformants. When one which expresses a mutant AKIII is selected among the aforementioned transformants, such a transformant also harbors a mutant gene.

Alternatively, it is also acceptable that a microorganism which produces a wild type enzyme is subjected to a mutation treatment to create a mutant strain which produces a mutant enzyme, and then a mutant gene is obtained from the mutant strain. The agent for performing a direct mutation treatment of DNA is exemplified by hydroxylamine and the like. Hydroxylamine is a chemical mutation treatment agent which causes mutation from cytosine to thymine by changing cytosine to N^4-hydroxycytosine. Alternatively, when a microorganisms itself is subjected to a mutation treatment, the treatment is performed by ultraviolet light irradiation, or using a mutating agent usually used for artificial mutation such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG).

Any one is used as a donor microorganism for DNA containing the wild type AKIII gene or AKIII gene having another mutation described above, provided that it is a microorganism belonging to the genus Escherichia. Concretely, it is possible to utilize those described in a book written by Neidhardt et al. (Neidhardt, F. C. et al., *Escherichia coli* and *Salmonella Typhimurium*, American Society for Microbiology, Washington D. C., 1208, table 1). For example, an *E. coli* JM109 strain and an MC1061 strain are exemplified. When the AKIII gene is obtained from these strains, preparation of chromosomal DNA, preparation of a chromosomal DNA library and the like may be performed in the same manner as the preparation of the DDPS gene described above. As the host to be used for preparation of the library, it is preferable to use a strain entirely deficient in AKI, II and III such as an *E. coli* GT3 strain (available from *E. coli* Genetic Stock Center (Connecticut, United States)).

From the obtained chromosomal DNA library, a bacterial strain having a recombinant DNA of the AKIII gene is obtained as a strain in which the AKIII activity is increased, or a strain in which auxotrophy is complemented. Cellular extracts are prepared from candidate strains, and crude enzyme solutions are prepared therefrom to confirm the AKIII activity. The measurement procedure for the AKIII enzyme activity may be performed in accordance with a method of Stadtman et al. (Stadtman, E. R., Cohen, G. N., LeBras, G., and Robichon-Szulmajster, H., *J. Biol. Chem.*, 236, 2033 (1961)).

For example, when a mutant strain completely deficient in AK is used as a host, a DNA fragment containing an AKIII gene can be obtained by isolating a transformed strain which becomes capable of growing on a medium not containing L-lysine, L-threonine, L-methionine and diaminopimelic acid, or on a medium not containing homoserine and diaminopimelic acid, and recovering recombinant DNA from the bacterial strain.

When the AKIII gene is amplified from chromosomal DNA by means of the PCR method, DNA primers to be used for the PCR reaction can be properly prepared on the basis of, for example, a sequence known in *E. coli* (Cassan, M., Parsot, C., Cohen, G. N., and Patte, J. C., *J. Biol. Chem.*, 261, 1052 (1986)). However, primers which can amplify a region comprising 1347 bases coding for lysC gene is suitable, and for example, two primers having sequences defined in SEQ ID NO:5 and NO:6 are suitable.

The method for carrying out mutation such as replacement, insertion and deletion of amino acid residue(s) on the AKIII gene obtained as described above is exemplified by the recombinant PCR method, the site specific mutagenesis method and the like, in the same manner as the mutation treatment of the DDPS gene described above.

Further, according to chemical synthesis of an aimed gene, it is possible to introduce mutation or random mutation into an aimed site.

Further, a method is available in which DNA of the AKIII gene on chromosome or extrachromosomal recombinant DNA is directly treated with hydroxylamine (Hashimoto, T. and Sekiguchi, M. *J. Bacteriol.*, 159, 1039 (1984)). Alternatively, it is acceptable to use a method in which a bacterium belonging to the genus Escherichia having an AKIII gene on chromosome or extrachromosomal recombinant DNA is irradiated by ultraviolet light, or a method to perform a treatment with a chemical agent such as N-methyl-N'-nitrosoguanidine or nitrous acid.

With respect to a selection method for the mutant AKIII gene, a strain completely deficient in AK, for example, an *E. coli* GT3 strain is at first transformed with a recombinant DNA containing an AKIII gene having been subjected to the mutation treatment. Next, transformed strains are cultivated on a minimal medium such as M9 containing a considerable amount of L-lysine. Strains harboring recombinant DNA containing a wild type AKIII gene cannot synthesize L-threonine, L-isoleucine, L-methionine and diaminopimelic acid (DAP) and are suppressed in growth because only one AK is inhibited by L-lysine. On the contrary, the strain harboring recombinant DNA containing the mutant AKIII gene in which inhibition by L-lysine is desensitized should be capable of growth on the minimal medium added with the considerable amount of L-lysine. This phenomenon can be utilized to select a strain which is resistant in growth to L-lysine or AEC as an analog of L-lysine, that is a strain harboring recombinant DNA containing a mutant AKIII gene in which inhibition is desensitized.

The mutant gene thus obtained may be introduced as a recombinant DNA into a suitable microorganism (host), and expressed. Thus a microorganism can be obtained which harbors AKIII being desensitized feedback inhibition.

The host is preferably a microorganism belonging to the genus Escherichia, for which *E. coli* is exemplified.

Alternatively, a mutant AKIII gene fragment may be taken out from the recombinant DNA, and inserted into another vector to make use. The vector DNA which can be used in the present invention is preferably plasmid vector DNA, for which there are exemplified pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219 and pMW218. Besides, vectors of phage DNA can be also utilized.

Further, in order to express the mutant AKIII gene efficiently, another promoter which works in microorganisms such as lac, trp and PL may be ligated upstream from a DNA sequence coding for the mutant AKIII, or a promoter contained in the AKIII gene may be used as it is, or after amplifying it.

In addition, as described above, the mutant gene may be inserted into an autonomously replicable vector DNA,

inserted into a host, and allowed to be harbored by the host as extrachromosomal DNA such as plasmid. Alternatively, the mutant gene may be integrated into chromosome of a host microorganism by a method using transduction, transposon (Berg, D. E. and Berg, C. M., *Bio/Technol.*, 1, 417 (1983)), Mu phage (Japanese Patent Application Laid-open No. 2-109985) or homologous recombination (*Experiments in Molecular Genetics*, Cold Spring Harbor Lab. (1972)).

<3> Production of L-lysine According to the Present Invention

L-lysine can be efficiently produced by cultivating, in a preferred medium, the bacterium transformed by introducing the mutant DDPS gene obtained as described above and allowed to harbor AK which is desensitized feedback inhibition by L-lysine, producing and accumulating L-lysine in a culture thereof, and collecting L-lysine from the culture. Namely, L-lysine can be efficiently produced by allowing the bacterium belonging to the genus Escherichia to harbor both of the mutant DDPS and the mutant AKIII.

The bacterium belonging to the genus Escherichia harboring AK which is desensitized feedback inhibition by L-lysine is exemplified by bacteria belonging to the genus Escherichia transformed by integrating, into chromosomal DNA, a DNA coding for AKIII having mutation to desensitize feedback inhibition by L-lysine, or bacteria belonging to the genus Escherichia transformed by introducing, into cells, a recombinant DNA constructed by ligating the DNA with a vector DNA autonomously replicable in cells of bacteria belonging to the genus Escherichia. Further, AK in which feedback inhibition by L-lysine is desensitized may be a wild type AK which does not suffer feedback inhibition by L-lysine, or one to which such a wild type AK gene is introduced into a bacterium belonging to the genus Escherichia in the same manner. Further, a mutant strain of a bacterium belonging to the genus Escherichia, which has become to produce a mutant AKIII by means of a mutation treatment of cells of a bacterium belonging to the genus Escherichia, is also acceptable.

On the other hand, in order to achieve transformation by introducing the mutant DDPS gene into a bacterium belonging to the genus Escherichia, the mutant DDPS gene may be integrated into chromosomal DNA to achieve transformation, or transformation may be achieved by introducing, into cells, a recombinant DNA constructed by ligating the mutant DDPS gene with a vector DNA autonomously replicable in cells of bacteria belonging to the genus Escherichia.

When the both of the mutant DDPS gene and the mutant AKIII gene are introduced into a bacterium belonging to the genus Escherichia, the both mutant genes may be integrated into and harbored on chromosomal DNA of the bacterium belonging to the genus Escherichia, or they may be harbored on an identical plasmid or separated plasmids in cells as extrachromosomal DNA. When separated plasmids are used, it is preferable to use plasmids having a stable distribution mechanism to allow each of them to be compatibly harbored in the cell. Further, one of the mutant genes may be integrated into and harbored on chromosomal DNA, and the other mutant gene may be harbored on a plasmid in cells as extrachromosomal DNA, respectively. When the mutant DDPS gene and the mutant AKIII gene are introduced into a bacterium belonging to the genus Escherichia, any order of introduction of the both genes is acceptable.

The productivity of L-lysine can be further improved by enhancing a dihydrodipicolinate reductase gene of the bacterium belonging to the genus Escherichia in which the mutant DDPS gene and the mutant AKIII gene have been introduced. The productivity of L-lysine can be still further improved by introducing a diaminopimelate dehydrogenase gene originating from a coryneform bacterium into the bacterium belonging to the genus Escherichia in which the dihydrodipicolinate reductase gene has been enhanced. This diaminopimelate dehydrogenase gene should be enhanced. Alternatively, the productivity of L-lysine can be also improved in a similar degree by enhancing tetrahydrodipicolinate succinylase gene and a succinyldiaminopimelate deacylase gene instead of the introduction of the diaminopimelate dehydrogenase.

The enhancement of gene herein refers to enhancement in activity of an enzyme as an expression product of the gene per a cell. Concretely, there may be exemplified enhancement in copy number of the gene in a cell, enhancement in expression amount per the gene by using a promoter having a high expression efficiency, and introduction of mutation to enhance enzyme activity into the gene. In order to enhance the copy number of a gene in a cell, the gene is inserted into a vector autonomously replicable in bacteria belonging to the genus Escherichia, and a bacterium belonging to the genus Escherichia may be transformed with this vector. This vector is preferably a multi-copy type plasmid. Alternatively, the copy number may be increased by amplifying DNA integrated into chromosomal DNA by using Mu phage or the like. With respect to the use of the plasmid, when plasmids are used for introduction of the mutant DDPS gene and the mutant AKIII gene, such plasmids having a stable distribution mechanism are preferably used in which these plasmids are stably harbored in a cell together. Any order of introduction of the genes is acceptable.

A mechanism will be explained below in which the productivity of L-lysine can be improved in a stepwise manner by successively enhancing genes of the L-lysine biosynthesis system as described above. A biosynthesis system comprising a plurality of reactions can be compared to a liquid flowing through a plurality of conduits having different thicknesses connected in serial. Herein each conduit corresponds to an individual enzyme, and the thickness of the conduit corresponds to an enzyme reaction velocity. In order to increase the amount of the liquid flowing through the conduits, it is effective to thicken the thinnest pipe. No effect can be expected even if a thick conduit is further thickened. In order to further increase the flow amount, the second thinnest conduit may be thickened. From such a viewpoint, the present inventors have tried to enhance the L-lysine biosynthesis system. For this purpose, as shown in Example 6 described below, the order of rate determining steps of the L-lysine biosynthesis system has been elucidated by introducing, into *E. coli*, genes of the L-lysine biosynthesis system originating from *E. coli* in a stepwise manner. In this elucidation, four genes of dapC succinyldiaminopimelate transaminase dapD (tetrahydrodipicolinate succinylase gene) dapE (succinyldiaminopimelate deacylase gene), and dapF (diaminopimelate epimerase gene) located downstream in the biosynthesis pathway were replaced with a gene DDH coding for DDH (diaminopimelate dehydrogenase) of *Brevibacterium lactofermentum* capable of catalyzing reactions participated by these gene products by itself. Namely, introduced genes for enzymes of the L-lysine biosynthesis system and the enzymes encoded by them are as follows:

ppc: phosphoenolpyruvate carboxylase

aspC: aspartate aminotransferase

lysC: aspartokinase III

lysC*: inhibition-desensitized aspartokinase III

asd: aspartate semialdehyde dehydrogenase

dapA: dihydrodipicolinate synthase

dapA*: inhibition-desensitized dihydrodipicolinate synthase

dapB: dihydrodipicolinate reductase

DDH: diaminopimelate dehydrogenase (originating from *Brevibacterium lactofermentum*)

lysA: diaminopimelate decarboxylase

As a result of individual introduction of each of the genes into *E. coli*, production of L-lysine was found in strains in which lysC*, dapA or dapA* was introduced, and a dapA*-introduced strain showed the highest L-lysine productivity. According to the result, it was found that a reaction catalyzed by dapA was the first rate determining step. Next, when each of the genes of the L-lysine biosynthesis system was introduced into the dapA*-introduced strain, lysC* had the largest effect on the improvement in L-lysine productivity. Thus it was found that a reaction catalyzed by lysC was the second rate determining step. In the same manner, it was found that a reaction catalyzed by dapB was the third rate determining step, and a reaction catalyzed by DDH was the fourth rate determining step. Further, as a result of investigation on rate determining steps among reactions catalyzed by dapC, dapD, dapE and dapF replaced with DDH, it was found that dapD and dapE concerned rate determining.

A method for obtaining the genes of the L-lysine biosynthesis system of *E. coli* and the DDH gene of Brevibacterium lactofermentum will be exemplified below.

The ppc gene can be obtained from a plasmid pS2 (Sabe, H et al., *Gene*, 31, 279 (1984)) or pT2 having this gene. A DNA fragment containing the ppc gene is obtained by cutting pS2 with AatII and AflII. A DNA fragment having the ppc gene is also obtained by cutting pT2 with SmaI and ScaI. An *E. coli* F15 strain (AJ12873) harboring pT2 is internationally deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology (postal code: 305, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) under a deposition number of FERM BP-4732 based on the Budapest Treaty.

The aspc gene is obtained from a plasmid pLF4 (Inokuchi, K et al., *Nucleic Acids Res*, 10, 6957 (1982)) having this gene. A DNA fragment having the aspc gene is obtained by cutting pLF4 with PvuII and StuI.

The asd gene is obtained from a plasmid pAD20 (Haziza, C. et al., *EMBO*, 1, 379 (1982)) having this gene. A DNA fragment having the asd gene is obtained by cutting pAD20 with AseI and ClaI.

The dapB gene is obtained by amplifying chromosomal DNA of *E. coli* by means of the PCR method by using two species of oligonucleotide primers (for example, SEQ ID NO:9, NO:10) prepared on the basis of a nucleotide sequence of a known dapB gene (Bouvier, J. et al., *J. Biol. Chem.*, 259, 14829 (1984)).

The DDH gene is obtained by amplifying chromosomal DNA of *Brevibacterium lactofermentum* by means of the PCR method by using two species of oligonucleotide primers (for example, SEQ ID NO:11, NO:12) prepared on the basis of a known nucleotide sequence of a DDH gene of *Corynebacterium glutamicum* (Ishino, S et al., *Nucleic Acids Res*, 15, 3917 (1987)).

The lysA gene is obtained by amplifying chromosomal DNA of *E. coli* by means of the PCR method by using two species of oligonucleotide primers (for example, SEQ ID NO:13, NO:14) prepared on the basis of a nucleotide sequence of a known lysA gene (Stragier, P. et al., *J. Mol. Biol.*, 168, 321 (1983))

The dapD gene is obtained by amplifying chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (for example, SEQ ID NO:15, NO:16) prepared on the basis of a nucleotide sequence of a known dapD gene (Richaud, C. et al., *J. Biol. Chem*, 259, 14824 (1984))

The dapE gene is obtained by amplifying *E. coli* DNA by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:17, NO:18) prepared on the basis of a nucleotide sequence of a known dapE gene (Bouvier, J. et al., *J. Bacteriol.*, 174, 5265 (1992)).

The dapF gene is obtained by amplifying chromosomal DNA of *E. coli* by means of the PCR method by using two species of oligonucleotide primers (for example, SEQ ID NO:19, NO:20) prepared on the basis of a nucleotide sequence of a known dapF gene (Richaud, C. et al., *Nucleic Acids Res*, 16, 10367 (1988))

In the present invention, any bacterium belonging to the genus Escherichia is available for the use as a host provided that a promoter of the mutant DDPS gene, the mutant AKIII gene or another gene of the L-lysine biosynthesis system, or another promoter for expressing these genes functions in its cells, and a replication origin of a vector DNA to be used for introduction functions in its cells to be capable of replication when the mutant DDPS gene, the mutant AKIII gene or another gene of the L-lysine biosynthesis system is introduced into a plasmid as extrachromosomal DNA.

For example, there may be exemplified L-lysine-producing *E. coli*, concretely a mutant strain having resistance to L-lysine analogs. The lysine analog is such one which inhibits proliferation of bacteria belonging to the genus Escherichia, but the suppression is entirely or partially desensitized if L-lysine co-exists in a medium. For example, there are oxalysine, lysine hydroxamate, AEC, γ-methyllysine, α-chlorocaprolactam and the like. Mutant strains having resistance to these lysine analogs are obtained by applying an ordinary artificial mutation operation to microorganisms belonging to the genus Escherichia. The bacterial strain to be used for L-lysine production is concretely exemplified by *Escherichia coli* AJ11442 (deposited as FERM BP-1543 and NRRL B-12185; see Japanese Patent Application Laid-open No. 56-18596 or U.S. Pat. No. 4,346, 170). In aspartokinase of the microorganisms described above, feedback inhibition by L-lysine is desensitized.

Besides, for example, L-threonine-producing microorganisms are exemplified, because inhibition of their aspartokinase by L-lysine is generally desensitized also in the L-threonine-producing microorganisms. As an L-threonine-producing bacterium belonging to *E. coli*, a B-3996 strain has the highest producibility known at present. The B-3996 strain is deposited in Research Institute for Genetics and Industrial Microorganism Breeding under a registration number of RIA 1867.

The medium to be used for cultivation of the transformant harboring the mutant gene according to the present invention is an ordinary medium containing a carbon source, a nitrogen source, organic ions and optionally other organic components.

As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose, or starch hydrolysate; alcohols such as glycerol or sorbitol; or organic acids such as fumaric acid, citric acid or succinic acid.

As the nitrogen source, it is possible to use inorganic ammonium salts such as ammonium sulfate, ammonium chloride or ammonium phosphate; organic nitrogen such as soybean hydrolysate; ammonia gas; or aqueous ammonia.

It is desirable to allow required substances such as vitamin B_1 and L-isoleucine or yeast extract to be contained in

appropriate amounts as organic trace nutrients. Other than the above, potassium phosphate, magnesium sulfate, iron ion, manganese ion and the like are added in small amounts, if necessary.

Cultivation is preferably carried out under an aerobic condition for 16–72 hours. The cultivation temperature is controlled at 25° C. to 45° C., and pH is controlled at 5–8 during cultivation. Inorganic or organic, acidic or alkaline substances as well as ammonia gas or the like can be used for pH adjustment.

Collection of L-lysine from a fermented liquor is usually carried out by combining an ion exchange resin method, a precipitation method and other known methods.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows preparation steps for pdapA1 and pdapA2.

FIG. 2 shows inhibition by L-lysine for wild type and mutant DDPS's.

FIG. 3 shows preparation steps for a plasmid pdapAS824 having a double mutation type dapA* gene.

FIG. 4 shows preparation steps for pLYSC1 and pLYSC2.

FIG. 5 shows an appearance ratio and a mutation ratio of transformants after a hydroxylamine treatment.

FIG. 6 shows inhibition by L-lysine for wild type and mutant AKIII's.

FIG. 7 shows preparation steps for a plasmid RSF24P originating from RSF1010 having dapA*24.

FIG. 8 shows preparation steps for a plasmid pLLC*80.

FIG. 9 shows preparation steps for a plasmid RSFD80 originating from RSF1010 having dapA*24 and lysC*80.

FIG. 10 shows structures of plasmids pdapA and pdapA* having dapA or dapA*.

FIG. 11 shows structures of plasmids plysC and plysC* having lysC or lysC*80.

FIG. 12 shows a structure of a plasmid pppc having ppc.

FIG. 13 shows a structure of a plasmid paspc having aspc.

FIG. 14 shows a structure of a plasmid pasd having asd.

FIG. 15 shows a structure of a plasmid pdapB having dapB.

FIG. 16 shows a structure of a plasmid pDDH having DDH.

FIG. 17 shows a structure of a plasmid plysA having lysA.

FIG. 18 shows preparation steps for a plasmid pCAB1 originating from RSF1010 having dapA*24, lysC*80 and dapB.

FIG. 19 shows preparation steps for a plasmid pCABD2 originating from RSF1010 having dapA*24, lysC*80, dapB and DDH.

FIG. 20 shows a structure of a plasmid pdapD having dapD.

FIG. 21 shows a structure of a plasmid pdapE having dapE.

FIG. 22 shows a structure of a plasmid pdapF having dapF.

FIG. 23 shows preparation steps for a plasmid pMW-dapDE1 having dapD and dapE.

FIG. 24 shows preparation steps for a plasmid pCABDE1 having dapA*24, lysC*80, dapB, dapD and dapE.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will be more concretely explained below with reference to Examples.

EXAMPLE 1

Preparation of Mutant DDPS Gene

<1> Cloning of Wild Type dapA Gene

A nucleotide sequence of a dapA gene of *E. coli* has been already reported (Richaud, F. et al., *J. Bacteriol.*, 297 (1986)), and it is known that its open reading frame (ORF) comprises 876 base pairs, and codes for a protein comprising 292 amino acid residues. Since it is unknown how this dapA gene is regulated, a region containing only an SD sequence and ORF except for a promoter region was amplified by using the PCR method and cloned.

Total genomic DNA of an *E. coli* K-12 MC1061 strain was extracted in accordance with a method of Saito and Miura (*Biochem. Biophys. Acta.*, 72, 619 (1963)). Two species of primers having sequences shown in SEQ ID NO:1 and NO:2 were prepared, which were used to perform the PCR reaction in accordance with a method of Erlich et al. (*PCR Technology*, Stockton press (1989)), and target DNA was amplified. Obtained DNA was inserted into a commercially available cloning vector pCR1000 for PCR fragments (purchased from Invitrogen, Ltd., (California, the United States)) as it was. pCR1000 contains a lacZ promoter (Placz), and is sold in a state of being cut at a site downstream from the lacZ promoter. When a recombinant DNA obtained by ligating a PCR fragment between both cut termini of pCR1000 is introduced into *E. coli*, the PCR fragment is transcribed under control of the lacZ promoter. Upon ligation of the PCR fragment with PCR1000, two species of plasmids were obtained, which were pdapA1 as a plasmid ligated in a normal orientation and pdapA2 as a plasmid ligated in a reversed orientation, for the direction of transcription of dapA with respect to the direction of transcription by the lacZ promoter (FIG. 1).

When these plasmids were introduced into *E. coli* JE7627 which is a strain deficient in DDPS, strains with the introduced plasmids is complemented auxotrophy for diaminopimelic acid of the host JE7627. Thus it was confirmed that DNA fragments inserted into the both plasmids contain the gene dapA coding for active DDPS.

A transformed strain obtained by introducing pdapA1 into a wild type *E. coli* W3110 strain (available from National Institute of Genetics (Mishima-shi, Shizuoka-ken, Japan)) was designated as W3110/pdapA1, and a transformed strain obtained by introducing pdapA2 into the *E. coli* W3110 strain was designated as W3110/pdapA2, respectively. These two transformed strains were cultivated respectively in a minimal medium M9 having the following composition added with AEC as an analog of lysine. The W3110 strain with no introduced plasmid was also cultivated in the same medium as a control. These two transformed strains and the W3110 strain having no plasmid were suppressed in growth by AEC, however, their growth inhibition was recovered by addition of L-lysine.

(Minimal medium M9)

A:	(20 × M9)	
	$Na_2HPO_4 \cdot 12H_2O$	303 g/L
	KH_2PO_4	60 g/L
	NaCl	10 g/L
	NH_4Cl	20 g/L

B: 1 M $MgSO_4$

C: 50% Glucose

D: 1 g/L Thiamine

A, B, C and D described above were separately sterilized, and mixed in a ratio of A:B:C:D: water=5:0.1:1:0.1:95

<2> Preparation of Mutant DDPS Gene (dapA*)

It was assumed that a strain harboring a plasmid containing dapA* coding for DDDPS with desensitized inhibition by L-lysine could grow on a minimal medium M9 added with a considerable amount of AEC. A strain harboring a plasmid containing dapA* was selected by their growth resistance to AEC.

In order to efficiently obtain dapA*, dapA's on pdapA1 and pdapA2 prepared in <1> were subjected to a mutation treatment.

(1-2-1) Investigation on Selection Condition for Strain Harboring Plasmid Containing dapA*

The W3110/pdapA1 strain and the W3110/pdapA2 strain obtained as described above were cultivated on M9 agar plate media containing various concentrations of AEC, respectively. Growth inhibitory concentrations by AEC were examined, and a selection condition was investigated for a strain harboring a plasmid containing dapA*.

Growth of the transformants on the M9 media containing AEC at various concentrations is shown in Table 1. In this table, +indicates growth of transformant, and−indicates no growth.

TABLE 1

AEC concentration (mM)	W3110/pdapA1	W3110/pdapA2
250	−	−
125	−	−
60	−	−
30	−	−
15	+	−
8	+	+
4	+	+
2	+	+

The direction of transcription of the dapA gene on pdapA1 coincides with the direction of transcription by the lacZ promoter (FIG. 1). Thus it was found that the dapA gene on pdapA1 provided resistance to AEC at considerably high concentrations even when dapA remained as a wild type because its expression amount was amplified by the lacZ promoter, while the dapA gene on pdapA2 had a smaller expression amount and provided inhibition in growth by AEC at lower concentrations because the direction of transcription was in the reversed direction with respect to the lacZ promoter, and a promoter of dapA's own was also deficient (the growth was suppressed in an allotment of addition of 30 mM in the case of the W3110/pdapA1 strain, and of 15 mM in the case of the W3110/pdapA2 strain). It was confirmed that the growth inhibition was eliminated by simultaneous addition of L-lysine.

Therefore, pdapA2 was used as an object for introduction of mutation. A medium prepared by adding 60 mM of AEC to the minimal medium M9 was used for selection of a strain harboring a plasmid containing dapA*. This medium is referred to as "selection medium" in Example 1 below.

(1-2-2) In Vitro Mutation Treatment for pdapA2 with Hydroxylamine

An in vitro mutation treatment method in which plasmids are directly treated with hydroxylamine was used for introduction of mutation into the pdapA2 plasmid.

2 μg of DNA was treated at 75° C. for 1–4 hours in 0.4 M hydroxylamine (0.1 M KH_2PO_4-1 mM EDTA (pH 6.0): 100 μl, 1 M hydroxylamine-1 mM EDTA (pH 6.0): 80 μl, DNA: 2 μg, total: 200 μl by filling up with water). DNA after the treatment was purified with glass powder, introduced into *E. coli* W3110, and spread on a complete medium (L-broth: 1% Bacto trypton, 0.5% Yeast extract, 0.5% NaCl, 1.5% agar), and colonies were formed. They were replicated onto the selection medium described in (1-2-1), and those which formed colonies on the selection medium were selected. Candidates of mutant plasmids in a total of 36 strains were obtained after two times of experiments.

The candidate strains of 36 strains in total thus obtained were spotted on the selection medium again, and AEC resistance was confirmed.

(1-2-3) Isolation of dapA* Gene and Investigation on dapA* Product

Mutant pdapA2's were recovered from the 36 strains described above. A dapA-deficient strain, JE7627 was transformed with them and the wild type pdapA2, respectively. A cell-free extract was prepared from each of the transformed strains, and the enzyme activity of DDPS was measured.

The cell-free extract (crude enzyme solution) was prepared as follows. A transformed strain was cultivated in a 2×TY medium (1.6% Bacto trypton, 1% Yeast extract, 0.5% NaCl), and collected at an optical density at 660 nm (OD_{660}) of about 0.8. A cell pellet was washed with 0.85% NaCl under a condition of 0° C., and suspended in 20 mM potassium phosphate buffer (pH 7.5) containing 400 mM KCl. The cells were ruptured by sonication (0° C., 200 W, 10 minutes). A ruptured cell solution was centrifuged at 33 krpm for 1 hour under a condition of 0° C. to obtain a supernatant to which ammonium sulfate was added to give 80% saturation to be stored at 0° C. overnight followed by centrifugation. A pellet was dissolved in 20 mM potassium phosphate buffer (pH 7.5)-400 mM KCl.

The enzyme activity of DDPS was measured in accordance with a method of Yugari et al. (Yugari, Y. and Gilvarg, C., *J. Biol. Chem*, 240, 4710 (1962)). Namely, the absorbance of a reaction solution having the following composition was measured at 37° C. with a spectrophotometer at a wavelength of 270 nm in a time-dependent manner. And generated dihydrodipicolinate was measured. Sodium pyruvate was removed from the reaction system to be used as a blank.

(Composition of Reaction Solution)

50 mM imidazole-HCl pH 7.4
20 mM L-aspartate semialdehyde
20 mM sodium pyruvate
enzyme solution
water (balance)
total 1.0 ml

Various concentrations of L-lysine were added to the enzyme reaction solution during measurement of the enzyme activity of DDPS, and the degree of inhibition by L-lysine was examined. As shown in FIG. 2, the wild type DDPS suffered inhibition by L-lysine. Mutant plasmids originating from the transformed strains having DDPS difficult to suffer inhibition by L-lysine as compared with the wild type were three species among the 36 species of the candidate plasmids. They were designated as pdapAS8, pdapAS9 and pdapAS24, respectively. According to following determination of nucleotide sequences, it was revealed that pdapAS8 and pdapAs9 had the same mutation.

The degree of desensitization of inhibition by L-lysine was varied in the three species of mutant DDPS encoded by pdapAS8, pdapAS9 and pdapAS24, however, the inhibition by L-lysine was desensitized in all of the three species. Although the specific activity of the enzyme might be affected by growth situations of cells and preparation of samples, it was found to be lowered a little in any case as compared with the wild type. However, it was judged that no substantial problem would be caused by them as a material for breeding.

(1-2-4) Determination of Nucleotide Sequence of Mutant dapA Gene

Nucleotide sequences of the mutant dapA genes were determined in accordance with an ordinary method by using a DNA sequencer ABI Model 373A (produced by Applied Biosystems Inc.). As a result, it was revealed that 487th C was changed to T in pdapAS8 and pdapAS9, and 597th C was changed to T in pdapAS24 on a sequence of the wild type dapA gene shown in SEQ ID NO:3. Therefore, it was revealed that a 81st alanine residue was changed to a valine residue in DDPS encoded by pdapAS8 and pdapAS9, and a 118th histidine residue was changed to a tyrosine residue in DDPS encoded by pdapAS24 in an amino acid sequence of DDPS shown in SEQ ID NO:4.

(1-2-5) Preparation of dapA Having Double Mutation

Two species of the mutant dapA genes were obtained as described above. In order to verify whether or not desensitization of inhibition works additively for these mutations, a plasmid containing mutant dapA having both of the two mutations was prepared. A procedure of preparation is as shown in FIG. 3. An obtained plasmid having double mutation was designated as pdapAS824.

EXAMPLE 2

Preparation of Mutant AKIII Gene

<1> Cloning of Wild Type lysC Gene

A nucleotide sequence of an AKIII gene (lysC) of *E. coli* has been already reported (Cassan, M., Parsot, C., Cohen, G. N., and Patte, J. C., *J. Biol Chem*, 261, 1052 (1986)), and it is known that its open reading frame (ORF) comprises 1347 base pairs, and codes for a protein comprising 449 amino acid residues. An operator is present in this gene, and is subjected to suppression by L-lysine. Thus in order to remove the operator region, a region containing only an SD sequence and ORF was amplified by using the PCR method and cloned.

Total genomic DNA of an *E. coli* K-12 MC1061 strain was prepared in accordance with a method of Saito and Miura (*Biochem Biophys Acta*, 72, 619 (1963)). Two species of primers having sequences shown in SEQ ID NO:5 and NO:6 were prepared, which were used to perform the PCR reaction in accordance with a method of Erlich et al (*PCR Technology*, Stockton press (1989)), and the lysc gene was amplified. Obtained DNA was digested with BamHI and AseI, then blunt-ended, and inserted into a SmaI site of a multi-copy vector, pUC18. This SmaI site is located at a downstream side from a lacZ promoter existing in the vector, and when recombinant DNA obtained by inserting a DNA fragment into the SmaI site of pUC18 is introduced into *E. coli*, the inserted DNA fragment is transcribed by means of read-through transcription under the control by the lacZ promoter. Upon insertion of the PCR fragment into the SmaI site of pUC18, two species of plasmids were obtained, which were pLYSC1 as a plasmid inserted in a reversed orientation and pLYSC2 as a plasmid inserted in a normal orientation, for the direction of transcription of lysC with respect to the direction of transcription by the lacZ promoter (FIG. 4).

When these plasmids were used to transform *E. coli* GT3 (thrA1016b, metLM1005, lysC1004) as a completely deficient strain for AKI, II, III, auxotrophy of GT3 for homoserine and diaminopimelic acid was complemented. Thus it was confirmed that DNA fragments inserted into the both plasmids contain the gene lysC coding for active AKIII.

A transformed strain obtained by introducing pLYSC1 into the AK completely deficient strain, *E. coli* GT3 was designated as GT3/pLYSC1, and a transformed strain obtained by introducing pLYSC2 into the *E coli* GT3 was designated as GT3/pLYSC2. A considerable amount of L-lysine was added to the minimal medium M9, and the GT3/pLYSC1 strain and the GT3/pLYSC2 strain were cultivated, respectively. Both of the GT3/pLYSC1 strain and the GT3/pLYSC2 strain harbor plasmids containing the wild type lysC, in which AKIII encoded by lysC on the plasmids is a sole AK. The wild type AKIII as the sole AK is inhibited by L-lysine in the presence of a considerable amount of L-lysine. Thus the both strains could not synthesize L-threonine, L-isoleucine, L-methionine and diaminopimelic acid (DAP), and were suppressed in growth.

<2> Preparation of Mutant AKIII Gene (lysC*)

It was assumed that a strain harboring a plasmid containing lysc* coding for AK with desensitized inhibition by L-lysine could grow on a minimal medium M9 added with a considerable amount of L-lysine. A strain harboring a plasmid containing lysC* was selected by selecting strains with their growth resistant to L-lysine or AEC as an analog of L-lysine.

In order to efficiently obtain lysc*, lysc's on pLYSC1 and pLYSC2 prepared in <1> were subjected to a mutation treatment.

(2-2-1) Investigation on Selection Condition for Strain Harboring Plasmid Containing lysC*

The GT3/pLYSC1 strain and the GT3/pLYSC2 strain were cultivated on M9 agar plate media containing various concentrations of L-lysine or AEC, respectively. Growth inhibitory concentrations by L-lysine or AEC were examined, and a selection condition was investigated for a strain harboring a plasmid containing lysC*.

Growth of the transformants on the M9 media containing L-lysine or AEC at various concentrations is shown in Table 2. In this table, + indicates growth of transformant, ± indicates a little growth, and – indicates no growth.

TABLE 2

	Growth and L-lysine concentration												
	0	0.2	0.4	0.8	1.5	3	6	12	25	50	100	200	(mM)
GT3/pLYSC1	+	–	–	–	–	–	–	–	–	–	–	–	
GT3/pLYSC2	+	+	+	+	+	+	+	+	+	+	+	–	

	Growth and AEC concentration										
	0	0.2	0.4	0.8	1.5	3	6	12	25	50	(mM)
GT3/pLYSC1	+	–	–	–	–	–	–	–	–	–	
GT3/pLYSC2	+	±	±	±	±	±	–	–	–	–	

The direction of transcription of the lysC gene on pLYSC2 coincides with the direction of transcription by the

lacZ promoter (FIG. 4). Thus it was found that the lysc gene on pLYSC2 provided resistance to L-lysine and AEC at considerably high concentrations even when lysC remained as a wild type because its expression amount was amplified by the lacZ promoter, while the lysC gene on pLYSC1 had a smaller expression amount and provided inhibition in growth by L-lysine and AEC at lower concentrations because the direction of transcription was in the reversed direction with respect to the lacZ promoter, and a promoter of itself was also deficient (the growth was not suppressed up to an allotment of addition of 100 mM for L-lysine and up to an allotment of addition of 3 mM for AEC in the case of the GT3/pLYSC2 strain, while the growth was completely suppressed in an allotment of addition of 0.2 mM for both L-lysine and AEC in the case of GT3/pLYSC1 strain). It was confirmed that the growth inhibition was eliminated by simultaneous addition of homoserine and diaminopimelic acid.

Therefore, pLYSC1 was used for experiments of introduction of mutation. A medium prepared by adding 10 mM of L-lysine or 0.2 mM of AEC to the minimal medium M9 was used for selection of plasmid-harboring strains containing lysC*. This medium is referred to as "selection medium" in Example 2 below.

(2-2-2) In Vitro Mutation Treatment for pLYSC1 with Hydroxylamine

Two kinds of methods were used for introduction of mutation into the pLYSC1 plasmid, which were an in vitro mutation treatment method in which plasmids are directly treated with hydroxylamine, and an additional in vivo mutation treatment method in which a cell harboring a plasmid is treated with nitrosoguanidine (NTG) followed by extraction of the plasmid in order to provide diversity of mutation, namely expecting mutation other than the mutation from cytosine to thymine with hydroxylamine.

(In Vitro Mutation Treatment with Hydroxylamine)

2 μg of DNA was treated under a condition of 75° C. for 1–4 hours in 0.4 M hydroxylamine (0.1 M KH_2PO_4-1 mM EDTA (pH 6.0): 100 μl, 1 M hydroxylamine-1 mM EDTA (pH 6.0): 80 μl, DNA: 2 μg, total: 200 μl by filling up with water). DNA after the treatment was purified with glass powder, introduced into an AK completely deficient strain, an *E. coli* GT3 strain, and spread on a complete medium (L-broth: 1% Bacto trypton, 0.5% Yeast extract, 0.5% NaCl, 1.5% agar), and colonies were formed. They were replicated onto the selection medium described in (2-2-1), and strains capable of growth on the selection medium were selected as candidate strains. The appearance ratio of transformants and the mutation ratio were found to proceed as shown in FIG. 5. Mutant strains were obtained by a treatment for 4 hours at a considerably high ratio of 0.5–0.8%.

(In Vivo Mutation Treatment with NTG)

pLYSC1 was introduced into *E. coli* MC1061, and an NTG treatment was performed with a whole cell. The cell after the treatment was cultivated overnight to fix mutation, and then a plasmid was extracted and introduced into *E. coli* GT3. Namely, the transformed strain was cultivated in a 2×TY medium (1.6% Bacto trypton, 1% Yeast extract, 0.5% NaCl), collected at an OD_{660} of about 0.3, washed with a TM buffer described below, then suspended in an NTG solution (prepared by dissolving NTG at a concentration of 0.2 mg/ml in TM buffer), and treated at 37° C. for 0–90 minutes. The cell was washed with TM buffer and 2×TY medium, and then mutation was fixed by cultivation in 2×TY medium overnight. Subsequently plasmid DNA was extracted from the cell, and introduced into an *E. coli* GT3 strain. Screening of candidate strains was performed in the same manner as in the in vitro mutation, and mutants of lysine resistance (Lys^R) and AEC resistance (AEC^R) were obtained.

(TM buffer)	
Tris	50 mM
Maleic acid	50 mM
$(NH_4)_2SO_4$	1 g/L
$MgSO_4 7H_2O$	0.1 g/L
$Ca(NO_3)2$	5 mg/L
$FeSO_4 7H_2O$	0.25 mg/L

pH was adjusted to 6.0 with NaOH.

Total 180 strains of candidate strains obtained as described above (hydroxylamine treatment: 48 strains, NTG treatment: 132 strains) were spotted on the selection medium again, and AEC and L-lysine resistances were confirmed to obtain 153 strains. Taking a notice of difference in amino acid accumulation pattern in the medium, these 153 strains were divided into 14 groups, and the AK activity was measured after selecting representative strains of each of the groups. There was no large difference in AK activity between the mutant strains obtained by the hydroxylamine treatment and the mutant strains obtained by the NTG treatment. Thus the following experiments were performed without distinguishing them.

(2-2-3) Isolation of lysc* Gene and Investigation on lysc* Product

No. 24, No. 43, No. 48, No. 60, No. 80, No. 117, No. 126, No. 149, No. 150, No. 156, No. 158, No. 167, No. 169 and No. 172 were selected as representative strains of the aforementioned 14 groups. Mutant plasmids derived from pLYSC1 were recovered from each of them, and designated as pLYSC1*24, pLYSC1*43, pLYSC1*48, pLYSC1*60, pLYSC1*80, pLYSC1*117, pLYSC1*126, pLYSC1*149, pLYSC1*150, pLYSC1*156, pLYSC1*158, pLYSC1*167, pLYSC1*169 and pLYSC1*172, respectively. An AK completely deficient strain GT3 was transformed with them and the wild type pLYSC1. A cell-free extract was prepared from each of transformed strains, and the enzyme activity of AKIII was measured.

The cell-free extract (crude enzyme solution) was prepared as follows. A transformed strain was cultivated in a 2×TY medium, and collected at an OD_{660} of about 0.8. Cells were washed with 0.02 M KH_2PO_4 (pH 6.75)-0.03 M β-mercaptoethanol under a condition of 0° C., and the cells were ruptured by sonication (0° C., 100 W, 30 minutes×4). A ruptured cell solution was centrifuged at 33 krpm for 1 hour under a condition of 0° C. to obtain a supernatant, to which ammonium sulfate was added to give 80% saturation. After centrifugation, a pellet was dissolved in 0.02 M KH_2PO_4 (pH 6.75)-0.03 M β-mercaptoethanol, and stored at 0° C. overnight.

The enzyme activity of AKIII was measured in accordance with a method of Stadtman et al. (Stadtman, E. R., Cohen, G. N., LeBras, G., and Robichon-Szulmajster, H., *J Biol. Chem.*, 236, 2033 (1961)). Namely, a reaction solution having the following composition was incubated at 27° C. for 45 minutes, and an $FeCl_3$ solution (2.8 N HCl 0.4 ml +12% TCA 0.4 ml +5% $FeCl_3$. $6H_2O$/0.1 N HCl 0.7 ml) was added to develop a color, which was centrifuged followed by measurement of absorbance of a supernatant at 540 nm. The activity was indicated by an amount of hydroxamic acid generated per minute (1 U=1 μmol/min). The molar absorption coefficient was 600. Potassium aspartate was removed from the reaction solution to be used as a blank.

(Composition of Reaction Solution)

Reaction mixture *1	0.3 ml
Hydroxylamine solution *2	0.2 ml
0.1 M Potassium aspartate (pH 7.0)	0.1 ml
Enzyme solution	
Water	(balance) total 1.0 ml

*1: 1 M Tris-HCl (pH 8.1) 9 ml + 0.3 M $MgSO_4$ 0.5 ml + 0.2 M ATP (pH 7.0) 5 ml
*2: 8 M Hydroxylamine solution was neutralized just before use with KOH.

Various concentrations of L-lysine were added to the enzyme reaction solution for measurement of the enzyme activity of AK, and the degree of inhibition by L-lysine was examined. Results are shown in FIG. 6 and Table 3. The wild type and Nos. 24, 43, 48, 60, 80, 117 and 126 are shown in FIG. 6A. Nos. 149, 150, 156, 158, 167, 169 and 172 are shown in FIG. 6B.

As shown in these results, the wild type AKIII strongly suffered inhibition by L-lysine, which was inhibited by 50% at about 0.45 mM of L-lysine, and inhibited by about 100% at 5 mM. On the contrary, the mutant AKIII's obtained this time had various degrees of desensitization, however, inhibition by L-lysine was desensitized in all of 14 species. Especially in the case of Nos. 24, 80, 117, 169 and 172, inhibition was scarcely observed even at 100 mM of L-lysine, and they had 50%-inhibitory-concentrations which were not less than 200 times as compared with that of the wild type. The specific activity per total protein, which might be affected by growth situations of cell and preparation of samples, was equal to or more than that of the wild type in almost all cases, in which there was little problem of decrease in activity due to the introduction of mutation (Table 3). According to this fact, it was postulated that an active center of AKIII was independent from a regulatory site by L-lysine with each other. In Table 3, the inhibition desensitization degree (%) refers to an AK activity in the presence of 100 mM of L-lysine with respect to an AK activity in the absence of L-lysine in the reaction solution. The heat stability (%) refers to a ratio of activity maintenance after a treatment at 55° C. for 1.5 hour.

TABLE 3

	Specific activity (U/mg protein)	Degree of desensitization of inhibition (%)*1	Heat stability (%)*2
Wild type	0.0247	0	18
No. 117	0.0069	120	0
No. 24	0.0218	100	30
No. 80	0.0244	99	36
No. 172	0.0189	97	0
No. 169	0.0128	96	2
No. 150	0.0062	77	25
No. 126	0.0250	61	39
No. 149	0.0256	59	9
No. 167	0.0083	43	45
No. 48	0.0228	38	42
No. 60	0.0144	35	9
No. 158	0.0224	22	42
No. 156	0.0101	18	2
No. 43	0.0212	17	0

*1: AK activity (%) in the presence of 100 mM of L-lysine with respect to AK activity in the absence of L-lysine
*2: ratio of activity maintenance (%) after treatment at 55° C. for 1.5 hour

Subsequently, the heat stability of the mutant enzymes was examined. When it is intended that an enzyme is improved to increase its activity, it is important that a created enzyme is maintained stably in cells. Measurement in vitro has some problems because of the difference in intracellular and extracellular protease activities and the influence of buffers for in vitro storage of enzymes. However, for convenience, the heat stability of the mutant AKIII's was investigated in vitro as one parameter.

Judging from results of investigation on the inactivation temperature of AKIII under various conditions, the ratio of activity maintenance after a treatment at 55° C. for 90 minutes was measured. As shown in Table 3, half the enzymes were rather more excellent than the wild type. Generally, a mutant enzyme is often unstable as compared with a wild type. However, some of the mutant AKIII's obtained this time were superior to the wild type in stability, and many of them seemed to be fairly useful in practical use for L-lysine production.

(2-2-4) Determination of Base Sequence of Wild Type lysC and Mutant lysC

A nucleotide sequence of the wild type lysC gene obtained this time was determined in accordance with an ordinary method by using a DNA sequencer ABI Model 373A (produced by Applied Biosystems Inc.) (SEQ ID NO:7). As a result, differences were found in six sites (two places at the amino acid level) from an already published sequence of lysc of an *E. coli* K-12 JC411 strain (Cassan, M., Rarsot, C., Cohen, G. N., and Patte, J. C., *J. Biol. Chem.*, 261, 1052 (1986)). It is speculated that the difference in six sites is due to the difference in bacterial strain used.

In the same manner, base sequences were determined for each of lysC*'s existing on the 14 species of mutant pLYSC1's, and mutation points were clarified. Results are shown in Table 4. In this table, indications in parentheses show mutations of amino acid residues based on mutations of nucleotides. Types of mutations were 12 kinds because two sets (No. 4 and No. 167, No. 24 and No. 80) had exactly the same mutation types among the 14 species. With respect to mutation types, Nos. 149, 150, 156, 158, 167, 169 and 172 were obtained by the hydroxylamine treatment, and Nos. 24, 43, 48, 60, 80, 117 and 126 were obtained by the NTG treatment. However, as for the pattern of mutation, any of them resided in mutation from cytosine to thymine, or mutation from guanine to adenine on a coding strand due to mutation from cytosine to thymine on a noncoding strand.

TABLE 4

Determination of mutation points of lysC*

lysC* mutation type	Mutagen	Mutation point (amino acid change)
No. 126	N	GGT→GA*T (^{323}Gly→Asp)
No. 43	N	GGT→GA*T (^{323}Gly→Asp) GGC→GA*C (^{406}Gly→Asp)
No. 149	H	CGT→T*GT (^{34}Arg→Cys) GGT→GA*T (^{323}Gly→Asp)
No. 48/167	N/H	CTC→T*TC (^{325}Leu→Phe)
No. 150	H	ATG→ATA* (^{318}Met→Ile)
No. 172	H	^{775}C→T (silent) ATG→ATA* (^{318}Met→Ile) GTG→A*TG (^{349}Val→Met)
No. 117	N	TCA→TT*A (^{345}Ser→Leu)
No. 158	H	GTG→A*TG (^{347}Val→Met)
No. 24/80	N/N	ACC→AT*C (^{352}Thr→Ile)
No. 169	H	^{923}C→T (silent) ACC→AT*C (^{352}Thr→Ile) TCT→TT*T (^{369}Ser→Phe)
No. 60	N	^{859}G→A (silent) GAA→A*AA (^{164}Glu→Lys)
No. 156	H	ATG→ATA* (^{417}Met→Ile) TGT→TA*T (^{419}Cys→Tyr) ^{2014}C→T (silent)

*: H; hydroxylamine treatment, N; NTG treatment

EXAMPLE 3

Fermentation Production of L-lysine with Strain Being Introduced daDA*

In order to produce L-lysine by using *E. coli*, as indicated in Japanese Patent Application Laid-open No. 56-18596, U.S. Pat. No. 4,346,170 and *Applied Microbiology and Biotechnology*, 15, 227–231 (1982), it is considered to be essential that a host for enhancing DDPS has an aspartokinase which is changed not to suffer inhibition by L-lysine. L-threonine-producing bacteria may be exemplified as such a strain. As for L-threonine-producing *E. coli*, a B-3996 strain has the highest productivity among those known at present. Thus the B-3996 strain was used as a host for evaluating dapA*. The B-3996 strain harbors pVIC40 extrachromosomally as a sole plasmid. Details are described in Japanese Patent Laid-open No. 3-501682 (PCT). This microorganism is deposited in Research Institute for Genetics and Industrial Microorganism Breeding under a registration No. of RIA 1867.

On the other hand, dapA* contained in pdapAS24 (in which the 118th histidine residue replaced with a tyrosine residue) was selected as dapA* to be introduced into *E. coli*, judging from the degree of desensitization of inhibition and the specific activity of the enzyme. At first, in order to increase the expression amount of dapA* and increase stability of the plasmid, mutant dapA* having existed on pdapAS24 (hereinafter referred to as "dapA*24") was ligated at the downstream from a promoter of a tetracycline resistance gene of pVIC40, and RSF24P was obtained as shown in FIG. 7.

A strain obtained by introducing the plasmid RSF24P into an *E. coli* JM109 strain was designated as AJ12395, which is deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology on October 28, 1993, as accession number of FERM P-13935, and transferred from the original deposition to international deposition based on Budapest Treaty on Nov. 1, 1994, and has been deposited as accession number of FERM BP-4858. Strains harboring pdapAS8 and pdapAS9 were not deposited. However, all of the mutation points of dapA* on each of the plasmids have been clarified as described above. Thus it is easy for those skilled in the art that the plasmid is recovered from the aforementioned deposited bacterium by using a method of Maniatis et al. (Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, 1.21 (1989)), and a target gene is obtained by using a site-directed mutagenesis method (Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, 15.63 (1989)).

pVIC40 was deleted from the B-3996 strain in accordance with an ordinary method, and a B-399 strain was obtained as a strain having no plasmid. The plasmid RSF24P was introduced into the B-399 strain in accordance with an ordinary method, and B-399/RSF24P was obtained. The L-lysine productivity of B-399/RSF24P was evaluated.

On the other hand, RSFP was constructed as a control plasmid. Namely, a large fragment was selected from digest of pVIC40 double-degested with BamHI and DraI as shown in FIG. 7, and it was blunt-ended with DNA polymerase Klenow fragment. The blunt-ended large fragment was self-ligated to obtain the plasmid RSFP. RSFP was introduced into the B-399 strain in accordance with an ordinary method, and B-399/RSFP was obtained. The L-lysine productivity was also evaluated for B-399/RSFP.

The cultivation was performed at an agitation of 114–116 rpm under a condition of a cultivation period of 48 hours and a temperature of 37° C. by using the following medium. Results are shown in Table 5.

(Medium for L-lysine Production)

A:	$(NH_4)_2SO_4$	16 g/L
	KH_2PO_4	1 g/L
	$MgSO_4 \cdot 7H_2O$	1 g/L
	$FeSO_4 \cdot 7H_2O$	0.01 g/L
	$MnSO_4 \cdot 5H_2O$	0.01 g/L
	Yeast Ext. (Difco)	2 g/L
	L-methionine	0.5 g/L
	L-threonine	0.1 g/L
	L-isoleucine	0.05 g/L
	pH is adjusted to 7.0 with KOH to be autoclave at 115° C. for 10 minutes.	(16/20 volume)
B:	20% Glucose (autoclave at 115° C. for 10 minutes)	(4/20 volume)
C:	Pharmacopoeial $CaCO_3$ (heat-sterilized in dry state at 380° C. for 2 days)	(30 g/L)

A and B are mixed in the ratio of A:B=4:1, 30 g of C is added to 1 L of the mixture and dissolved, and antibiotics (streptomycin: 100 μg/ml, kanamycin: 5 μg/ml) are added.

TABLE 5

Bacterial strain	Production amount of L-lysine hydrochloride
B-399/RSF24P	4.1 g/L
B-399/RSFP	0 g/L

EXAMPLE 4

Fermentation Production of L-lysine with Strain Being Introduced dapA* and lysC* (I)

The effect of the mutant DDPS on L-lysine production has been shown in Example 3. In order to achieve further improvement, the mutant AKIII gene obtained in Example 2 was allowed to co-exist with the mutant DDPS gene. The mutant AKIII gene to co-exist with the mutant DDPS gene was selected as originating from the No. 80 strain (lysC*80), judging from the enzyme activity, heat stability and the like.

lysC*80 was used after excising it from a plasmid pLLC*80 (FIG. 8) prepared by alternatively ligating lysC* having existed on pLYSC1*80 (hereinafter referred to as "lysC*80") at the downstream of a lacZ promoter of vector pHSG399 (produced by Takara Shuzo Co., Ltd.) which has an inverted-directional-insertion site with respect to pUC18 in order to increase the expression amount of lysC*. pLLC*80 is a plasmid prepared to arrange lysC*80 to allow the direction of transcription to have a normal orientation with respect to the lacZ promoter in order to improve the productivity of L-lysine because lysC*80 on pLYSC1*80 has its direction of transcription arranged in a reversed orientation with respect to the lacZ promoter.

A plasmid, RSFD80, having dapA* and lysC* was prepared from pLLC*80 and RSF24P obtained in Example 3 as shown in FIG. 9. RSFD80 includes dapA*24 and lysC*80 arranged in this order to allow the direction of transcription to have a normal orientation with respect to tetP at the downstream from a promoter (tetp) of a tetracycline resistance gene.

The RSFD80 μplasmid was introduced into an *E. coli* JM109 strain, which was designated as AJ12396. AJ12396 is deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology on Oct. 28, 1993, as accession number of FERM P-13936, and transferred from the original deposition to international deposition based on Budapest Treaty on Nov. 1, 1994, and has been deposited as accession number of FERM BP-4859.

Strains harboring pLYSC1*24, pLYSC1*43, pLYSC1*48, pLYSC1*60, pLYSC1*117, pLYSC1*126, pLYSC1*149, pLYSC1*150, pLYSC1*156, pLYSC1*158, pLYSC1*167, pLYSC1*169 and pLYSC1*172 were not deposited. However, all of the mutation points of lysC* on each of the plasmids have been clarified as described above. Thus it is easy for those skilled in the art that the plasmid is recovered from the aforementioned deposited bacterium by using a method of Maniatis et al. (Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, 1.21 (1989)), and a target gene is obtained by using a site-directed mutagenesis method (Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, 15.63 (1989)). RSFD80 was introduced into B-399 strain in accordance with an ordinary method, and B-399/RSFD80 was obtained. The L-lysine productivity of B-399/RSFD80 was evaluated. The L-lysine productivity was also evaluated for B-399/RSFP as a control.

The cultivation was performed at an agitation of 114–116 rpm under a condition of a cultivation period of 48 hours and a temperature of 37° C. by using the same medium for production of L-lysine as in Example 3. Results are shown in Table 6.

TABLE 6

Bacterial strain	Production amount of L-lysine hydrochloride
B-399/RSFD80	9.2 g/L
B-399/RSFP	0 g/L

EXAMPLE 5

Fermentation Production of L-lysine with Strain Being Introduced dapA* and lysC* (II)

It has been confirmed in Example 4 that the productivity of L-lysine can be improved by allowing the bacterium belonging to the genus Escherichia to harbor the mutant dapA gene and the mutant lysC gene. Experiments were performed to confirm whether or not this effect was maintained when the host is changed.

An *E. coli* W3110(tyrA) strain was used as a host. The W3110(tyrA) strain is described in detail in European Patent Publication No. 488424/92. Its preparation method will be briefly described as follows. The *E. coli* W3110 strain was obtained from National Institute of Genetics (Mishima-shi, Shizuoka-ken, Japan). This strain was spread on an LB plate containing streptomycin, and a streptomycin resistant strain was obtained by selecting strains which formed colonies. The selected streptomycin resistant strain was mixed with an *E. coli* K-12 ME8424 strain, and stationarily cultivated in a complete medium (L-Broth: 1% Bacto trypton, 0.5% Yeast extract, 0.5% NaCl) under a condition of 37° C. for 15 minutes to induce conjugation. The *E. coli* K-12 ME8424 strain has genetic characters of ($HfrPO_{45}$, thi, relA1, tyrA::Tn10, ung-1, nadB), which is available from National Institute of Genetics.

The culture was then spread on a complete medium (L-Broth: 1% Bacto trypton, 0.5% Yeast extract, 0.5% NaCl, 1.5% agar) containing streptomycin, tetracycline and L-tyrosine, and a colony-forming strain was selected. This strain was designated as *E. coli* W3110(tyrA) strain.

By the way, European Patent Publication No. 488424/92 describes many strains formed by introducing plasmids into the W3110(tyrA) strain. For example, a strain obtained by introducing a plasmid pHATerm is designated as *E. coli* W3110(tyrA)/pHATerm strain, and deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology, to which a registration No. of FERM BP-3653 is given. The W3110(tyrA) strain can be also obtained by curing the plasmid pHATerm from the *E. coli* W3110(tyrA)/pHATerm strain. The curing of the plasmid can be performed in accordance with an ordinary method.

The plasmid RSFD80 containing both of dapA* and lysc* obtained in Example 4 was introduced into the W3110(tyrA) obtained as described above, and W3110(tyrA)/RSFD80 was obtained. The L-lysine productivity was evaluated for W3110(tyrA)/RSFD80. As a control, RSFP was introduced into the W3110(tyrA) strain in accordance with an ordinary method, and W3110(tyrA)/RSFP was obtained. The L-lysine productivity was also evaluated for W3110(tyrA)/RSFP as a control.

The cultivation was performed at an agitation of 114–116 rpm under a condition of a cultivation period of 48 hours and a temperature of 37° C. by using the aforementioned medium for L-lysine production. Results are shown in Table 7.

TABLE 7

Bacterial strain	Production amount of L-lysine hydrochloride
W3110 (tyrA) /RSFD80	8.9 g/L
W3110 (tyrA) /RSFP	0 g/L

EXAMPLE 6

Analysis of Rate Determining Steps of L-lysine Biosynthesis System and Improvement in L-lysine Productivity of L-lysine-producing Bacteria Belonging to the Genus Escherichia

It was tried to improve the L-lysine productivity by analyzing rate determining steps of the L-lysine biosynthesis system of *E. coli* and enhancing genes for enzymes which catalyze the steps.

<1> Identification of the First Rate Determining Steps (6-1-1) Preparation of Genes of L-lysine Biosynthesis System

The rate determining step was identified by isolating various genes of the L-lysine biosynthesis system, introducing these genes into *E. coli*, and examining effects of each of the genes on the L-lysine productivity. The introduced genes for enzymes of the L-lysine biosynthesis system, and the enzymes encoded by them are as follows.

ppc: phosphoenolpyruvate carboxylase

aspc: aspartate aminotransferase

lysC: aspartokinase III

lysC*80: inhibition-desensitized aspartokinase III

asd: aspartate semialdehyde dehydrogenase

dapA: dihydrodipicolinate synthase

dapA*24: inhibition-desensitized dihydrodipicolinate synthase

dapB: dihydrodipicolinate reductase

DDH: diaminopimelate dehydrogenase (originating from *Brevibacterium lactofermentum*)

lysA: diaminopimelate decarboxylase

The L-lysine biosynthesis system from phosphoenolpyruvic acid to L-lysine can be thoroughly covered by the genes described above. The dapc, dapD, dapE and dapF genes, among the genes of the L-lysine biosynthesis system origi-

nally possessed by *E. coli*, are replaced with the gene DDH coding for DDH (diaminopimelate dehydrogenase) of *Brevibacterium lactofermentum* which can catalyze reactions concerning these gene products by itself. The W3110(tyrA) strain of the *E. coli* K-12 series was used as a host for introducing these genes.

The dapA and dapA*24 genes were respectively obtained by excision from pdapA2 and pdapAS24 (see Example 1) with EcoRI and KpnI (FIG. 10). These genes were ligated with pMW118 which was digested with EcoRI and KpnI to obtain pdapA and pdapA*. The lysC and lysC*80 genes were respectively obtained by excision from pLYSC1 and pLLC*80 (see Example 2) with EcoRI and SphI. These genes were ligated with pMW119 which was digested with EcoRI and SphI to obtain plysc and plysC* (FIG. 11).

The ppc gene was obtained from a plasmid pT2 having this gene. pT2 was cut with SmaI and ScaI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid pppc (FIG. 12). *E. coli* F15 (AJ12873) harboring pT2 is deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under an accession number of FERM BP-4732.

The aspC gene was obtained from a plasmid pLF4 (Inokuchi, K. et al., *Nucleic Acids Res.*, 10, 6957 (1982)) having this gene (FIG. 13). pLF4 was cut with PvuII and StuI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW119 to obtain a plasmid paspc.

The asd gene was obtained from a plasmid pAD20 (Haziza, C. et al., *EMBO*, 1, 379 (1982)) having this gene. pAD20 was cut with AseI and ClaI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid pasd (FIG. 14).

The dapB gene was obtained by amplifying a dapB gene from chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:9, NO:10) prepared on the basis of a nucleotide sequence of a known dapB gene (Bouvier, J. et al., *J. Biol. Chem.*, 259, 14829 (1984)) (FIG. 15). An obtained amplified DNA fragment was cut with AseI and DraI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW119 to obtain a plasmid pdapB.

The DDH gene was obtained by amplifying a DDH gene from chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:11, NO:12) prepared on the basis of a known nucleotide sequence of a DDH gene of *Corynebacterium glutamicum* (Ishino, S. et al., *Nucleic Acids Res.*, 15, 3917 (1987)). An obtained amplified DNA fragment was cut with EcoT22I and AvaI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW119 to obtain a plasmid pDDH (FIG. 16).

The lysA gene was obtained by amplifying a lysA gene from chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:13, NO:14) prepared on the basis of a nucleotide sequence of a known lysA gene (Stragier, P. et al., *J. Mol. Biol.*, 168, 321 (1983)). An obtained amplified DNA fragment was cut with SplI and BclI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid plysA (FIG. 17).

Confirmation of the fact that each of the aforementioned genes was cloned was performed by cutting them with restriction enzymes shown in the figures. The vectors pMW118 and pMW119 (produced by Nippon Gene) used for cloning of these genes were selected because they were able to co-exist in cells of *E. coli* together with RSF1010 as a vector used for preparation of plasmids for lysine production described below, and also had a stable distribution mechanism.

(6-1-2) L-lysine Productivity of *E. coli* with Introduced Genes of L-lysine Biosynthesis System

E. coli W3110(tyrA) was transformed with each of the plasmids containing the genes of the L-lysine biosynthesis system described above, and obtained transformants were cultivated to perform L-lysine production. The cultivation was performed for 30 hours under a condition of a cultivation temperature of 37° C. and an agitation of 114–116 rpm by using the following medium. Results are shown in Table 8.

(Medium Composition)

Glucose	40 g/l
$MgSO_4 \cdot 7H_2O$	1 g/l
$(NH_4)_2SO_4$	16 g/l
KH_2PO_4	1 g/l
$FeSO_4 \cdot 7H_2O$	0.01 g/l
$MnSO_4 \cdot 5H_2O$	0.01 g/l
Yeast Ext. (Difco)	2 g/l
L-tyrosine	0.1 g/l
pH was adjusted to 7.0 with KOH to be autoclaved at 115° C. for 10 minutes (Glucose and $MgSO_4 \cdot 7H_2O$ were separately sterilized)	
Pharmacopoeial $CaCO_3$ (heat-sterilized in dry state at 180° C. for 2 days)	25 g/l
Antibiotics (streptomycin 20 mg/l or ampicillin 50 mg/l depending on species of plasmids to be introduced)	

TABLE 8

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
W3110(tyrA)	0.08	0.2
W3110(tyrA)/pppc	0.08	0.2
W3110(tyrA)/paspC	0.12	0.3
W3110(tyrA)/plysC	0.08	0.2
W3110(tyrA)/plysC*	2.27	5.57
W3110(tyrA)/pasd	0.12	0.3
W3110(tyrA)/pdapA	2.32	5.70
W3110(tyrA)/pdapA*	3.63	8.90
W3110(tyrA)/pdapB	0.08	0.2
W3110(tyrA)/pDDH	0.08	0.2
W3110(tyrA)/plysA	0.12	0.3

i E. coliW3110(tyrA) became to produce L-lysine by introduction of plysC*, pdapA or pdapA*. Since both of lysC product and dapA product suffer feedback inhibition by L-lysine, it can be postulated that these enzymes are major regulatory points in L-lysine biosynthesis. The reaction catalyzed by dapA product exists in a position of branching to a biosynthesis system for L-threonine, L-methionine and L-isoleucine and a biosynthesis system for L-lysine, and is the first step of the biosynthesis system inherent to L-lysine. It was already reported that *E. coli* also becomes to produce L-lysine by amplification of a wild type dapA (Eur. J. Appl. Microbiol. Biotechnol., 15, 227 (1982)), which has been also confirmed from the result described above. On the other hand, the result of Example 3 has been confirmed again in that the yield of L-lysine is further increased when dapA* as an inhibition-desensitized type gene is introduced into *E. coli*.

Crude enzyme solutions were prepared from W3110 (tyrA), W3110(tyrA)/pdapA and W3110(tyrA)/pdapA* in the same manner as in Example 1, the DDPS (dihydrodipicolinate synthase) activity was measured, and the degree of inhibition of the DDPS activity by L-lysine was examined. Results are shown in Table 9.

TABLE 9

Bacterial strain	Specific activity *1	Degree of desensitization of inhibition *2
W3110(tyrA)	0.0423	50
W3110(tyrA)/pdapA	0.2754	22.9
W3110(tyrA)/pdapA*	0.1440	76.5

*1: μmols/min/mg protein
*2: ratio of activity maintenance (%) in the presence of 5 mM of L-lysine

The inhibition-desensitized dapA* product probably has a large effect on L-lysine production because it has a high degree of desensitization of inhibition although it has a lower specific activity than the wild type enzyme (about ½). The necessity of the desensitization of inhibition of the dapA product has been shown for L-lysine production.

In addition, the fact that lysc* has an effect on L-lysine production can be considered as follows. The first rate determining step is a step at which HD (homoserine dehydrogenase: product of thrA or metLM) competes with DDPS (dapA product) in acquiring ASA (aspartate-β-semialdehyde) as a substrate to serve at a branching point of the biosynthesis system, and when dapA is enhanced as described above, the reaction flows in a direction of L-lysine biosynthesis. On the other hand, it is speculated that when the supply amount of ASA is increased by enhancing lysc which participates in a reaction further upstream from dapA, any of reactions relevant to HD and DDPS is also facilitated, and thus the production amount of L-lysine has been also increased. However, this effect is scarcely obtained by enhancement of the wild type lysC only. This is probably because the inhibition of the wild type AKIII (lysC product) by L-lysine is more strict than that of the wild type DDPS (AKIII and DDPS are inhibited by about 100% and 80% respectively in the presence of 5 mM of L-lysine).

According to the facts described above, it was judged that the reaction by DDPS having a higher lysine-producing effect was the first rate determining step, and it was postulated that the reaction by AKIII was the second rate determining step.

<2> Identification of the Second Rate Determining Step

The second rate determining step was identified by enhancing various genes of the L-lysine biosynthesis system in strains with introduced dapA*. In order that other plasmids were stably harbored when they were introduced into *E. coli* harboring a plasmid containing dapA*, dapA* was transferred from pdapA to RSF1010, and RSF24P was obtained (FIG. 7). *E. coli* W3110(tyrA) was transformed with the plasmid RSF24P having dapA*.

Plasmids having genes of the L-lysine biosynthesis system were introduced into *E. coli* W3110(tyrA)/RSF24P. Two species of plasmids, namely RSF24P and a plasmid containing another gene of the L-lysine biosynthesis system, co-exist in each of obtained transformants. The L-lysine productivity was examined for these strains in the same manner as in (6-1-2). Results are shown in Table 10.

TABLE 10

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
W3110(tyrA)/RSF24P	3.63	8.9
W3110(tyrA)/RSF24P + pppc	3.67	9.0
W3110(tyrA)/RSF24P + paspC	3.59	8.8
W3110(tyrA)/RSF24P + plysC	3.42	8.4
W3110(tyrA)/RSF24P + plysC*	9.17	22.5
W3110(tyrA)/RSF24P + pasd	3.75	9.2
W3110(tyrA)/RSF24P + pdapA	3.55	8.7
W3110(tyrA)/RSF24P + pdapA*	3.46	8.5
W3110(tyrA)/RSF24P + pdapB	4.08	10.0
W3110(tyrA)/RSF24P + pDDH	3.67	9.0
W3110(tyrA)/RSF24P + plysA	3.55	8.7

As a result, a remarkable enhancing effect on the L-lysine productivity was found only in lysC*. The wild type lysC had no effect at all. This is probably because the inhibition by L-lysine is strong as described above. Thus it was confirmed that the reaction participated by lysC* was the second rate determining step.

lysc* was integrated into RSF24P, and RSFD80 was obtained (FIG. 9). In the same manner, lysC was integrated into RSF24P, and an obtained plasmid was designated as RSFD1. These plasmids were introduced into *E. coli* W3110 (tyrA), crude enzyme solutions were prepared, and the AK activity and the degree of inhibition of AK activity by L-lysine were examined in the same manner as in (6-1-2). Results are shown in Table 11.

TABLE 11

Bacterial strain for AK activity	Specific activity *1	Degree of desensitization of inhibition *2
W3110(tyrA)/RSF24P	0.94	42.9
W3110(tyrA)/RSFD1	18.55	7.2
W3110(tyrA)/RSFD80	33.36	98.8

*1: nmols/min/mg protein
*2: ratio of activity maintenance (%) in the presence of 5 mM of L-lysine

The specific activities of AK of the strains harboring the plasmids were increased 20–30 times by integrating lysC and lysC* into RSF24P. *E. coli* has three species of AK's, and lysC codes for AKIII among them. However, a total activity of the three species of AK's was measured in the experiment described above. It is speculated that the inhibition by L-lysine also becomes high in the strain harboring RSFD1 with the inserted wild type lysc because the ratio occupied by AKIII is higher than those by AKI and AKIII as compared with the control (W3110(tyrA)/RSF24P), resulting in no indication of the effect on enhancement of the L-lysine productivity. On the other hand, it was revealed that the inhibition was desensitized for about 100% of AKIII in the strain harboring RSFD80, and this fact contributed to the improvement in L-lysine production.

<3> Identification of the Third Rate Determining Step

Next, various plasmids of the L-lysine biosynthesis system were introduced into *E. coli* W3110(tyrA)/RSFD80, and cultivation for L-lysine production was performed. Cultivation results are shown in Table 12.

TABLE 12

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
W3110(tyrA)/RSFD80	9.17	22.5
W3110(tyrA)/RSFD80 + pppc	8.97	22.0
W3110(tyrA)/RSFD80 + paspC	9.05	22.2
W3110(tyrA)/RSFD80 + plysC	8.56	21.0
W3110(tyrA)/RSFD80 + plysC*	8.15	20.0
W3110(tyrA)/RSFD80 + pasd	8.35	20.5
W3110(tyrA)/RSFD80 + pdapA	8.56	21.0
W3110(tyrA)/RSFD80 + pdapA*	8.15	20.0
W3110(tyrA)/RSFD80 + pdapB	10.80	26.5
W3110(tyrA)/RSFD80 + pDDH	8.56	21.0
W3110(tyrA)/RSFD80 + plysA	8.48	20.8

An enhancing effect on the L-lysine productivity was observed only in dapB, and it was found that the reaction participated by dapB was the third rate determining step. Thus dapB was inserted into RSFD80, and pCAB1 was obtained (FIG. 18). This plasmid was introduced into *E. coli* W3110(tyrA), a crude enzyme solution was prepared, and the enzyme activity of DDPR (dihydrodipicolinate reductase) was measured in accordance with a method described by Tamir, H. and Gilvarg, C., *J. Biol. Chem.*, 249, 3034 (1974). In the same manner, crude enzyme solutions were prepared from a strain harboring only RSFD80 and a strain harboring both RSFD80 and pdapB, and the DDPR activity was measured. Results are shown in Table 13.

TABLE 13

Bacterial strain	Specific activity (μmols/min/mg protein)
W3110(tyrA)/RSFD80	0.027
W3110(tyrA)/RSFD80 + pdapB	0.092
W3110(tyrA)/PCAB1	0.178

The DDPR activity was increased about 3 times in the strain harboring RSFD80 and pdapB, and it was increased about 6.5 times in the strain harboring pCAB1 in which dapB was inserted into RSFD80, as compared with the control (strain harboring RSFD80 only). According to the fact that both W3110(tyrA)/RSFD80+pdapB and W3110 (tyrA)/pCAB1 had equivalent L-lysine accumulation of 10.8 g/l, it was judged that dapB was provided in a sufficient amount for L-lysine production, and the rate determining step was shifted to the next step.

<4> Identification of the Fourth Rate Determining Step

Next, the fourth rate determining step was identified by using the plasmid pCAB1 harboring lysc*, dapA* and dapB. Various plasmids of the L-lysine biosynthesis system were introduced into *E. coli* W3110(tyrA)/pCAB1, and cultivation for L-lysine production was performed. Cultivation results are shown in Table 14.

TABLE 14

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
W3110(tyrA)/pCAB1	10.80	26.5
W3110(tyrA)/pCAB1 + pppc	11.00	27.0
W3110(tyrA)/pCAB1 + paspC	10.88	26.7
W3110(tyrA)/pCAB1 + plysC	10.60	26.0
W3110(tyrA)/pCAB1 + plysC*	10.39	25.5
W3110(tyrA)/pCAB1 + pasd	10.19	25.0
W3110(tyrA)/pCAB1 + pdapA	10.72	26.3
W3110(tyrA)/pCAB1 + pdapA*	10.80	26.5
W3110(tyrA)/pCAB1 + pdapB	10.92	26.8
W3110(tyrA)/pCAB1 + pDDH	12.23	30.0
W3110(tyrA)/pCAB1 + plysA	10.60	26.0

An enhancing effect on the L-lysine productivity was observed only in DDH, and it was found that the reaction catalyzed by DDH was the fourth rate determining step. In addition, SDAP (N-succinyl-L,L-α, ε-diaminopimelic acid) detected in a culture broth of the DDH non-introduced strain was not detected in a culture broth of the DDH introduced strain. Detection of SDAP was performed by means of TLC development (composition of development solvent; methanol:water:10N HCl:pyridine =80:17.5:2.5:10) (Bouvier, J., Richaud, C., Higgins, W., Bogler, O. and Stragier, P., *J. Bacteriol.*, 174, 5265 (1992)). Further, the color of broth was brown in the case of the DDH non-introduced strain, but it was changed to yellow in the case of the DDH introduced strain. Thus DDH was integrated into pCAB1 to prepared a plasmid pCABD2 (FIG. 19), and the DDH activity of *E. coli* W3110(tyrA) transformed with this plasmid was measured. The DDH enzyme activity was measured in accordance with a literature (Azizono, Haruo, *Fermentation and Industry*, 45, 964 (1987)). Results are shown in Table 15.

TABLE 15

Bacterial strain	Specific activity (μmols/min/mg protein)
W3110(tyrA)/pCAB1	0.000
W3110(tyrA)/pCAB1 + pDDH	0.799
W3110(tyrA)/pCABD2	2.214

The DDH activity was not detected in the control (W3110 (tyrA)/pCAB1) because DDH was originally not present in *E. coli*. The specific activity of DDH of the strain harboring pCABD2 (W3110(tyrA)/pCABD2) was about 2.5 times that of the strain harboring pDDH (W3110(tyrA)/pCAB1+ pDDH), however, the both strain had an equivalent L-lysine accumulation amount (12.23 g/l). Thus it was judged that the DDH expression amount of pCABD2 was a sufficient amount.

<5> Analysis of Rate Determining Steps Among dapC, dapD, dapE and dapF

Next, in order to examine a rate limiting order of dapC, dapD, dapE and dapF replaced by DDH in the analysis described above, at first these genes were cloned. dapc was not cloned because of no report on its base sequence, however, the remaining three species of genes were cloned in accordance with the PCR method.

The dapD gene was obtained by amplifying a dapD gene from chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:15, NO:16) prepared on the basis of a nucleotide sequence of a known dapD gene (Richaud, C. et al., *J. Biol. Chem.*, 259, 14824 (1984)). An obtained amplified DNA fragment was cut with Eco0109I and SacI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid pdapD (FIG. 20).

The dapE gene was obtained by amplifying a dapE gene from chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:17, NO:18) prepared on the basis of a nucleotide sequence of a known dapE gene (Bouvier, J. et al., *J. Bacteriol.*, 174, 5265 (1992)). An obtained amplified DNA fragment was cut with MunI and BalII, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid pdapE (FIG. 21).

The dapF gene was obtained by amplifying a dapF gene from chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:19, NO:20) prepared on the basis of a nucleotide sequence of a known dapF gene (Richaud, C. et al., *Nucleic Acids Res.*, 16, 10367 (1988)). An obtained amplified DNA fragment was cut with PstI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid pdapF (FIG. 22).

Each of the plasmids obtained as described above was introduced into W3110(tyrA)/pCAB1, and cultivation for L-lysine production was performed. In the previous experiment, the changes were observed in the color of broth and in the presence or absence of accumulation of the intermediate (SDAP) in addition to the L-lysine production amount between before and after the introduction of DDH. Thus the analysis of the rate determining step was performed also by using them as indexes. Results are shown in Table 16.

TABLE 16

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)	Color of broth	Accumulation of SDAP
W3110(tyrA)/pCAB1	10.80	26.5	brown	+
W3110(tyrA)/pCAB1 + pdapD	11.08	27.2	yellow	+
W3110(tyrA)/pCAB1 + pdapE	11.12	27.3	brown	−
W3110(tyrA)/pCAB1 + pdapF	10.96	26.9	brown	+
W3110(tyrA)/pCABD2	12.23	30.0	yellow	−

The production amount of L-lysine was increased a little by the enhancement of dapD or dapE, but DDH was not exceeded. Further, it was found that the change in color of broth and the accumulation of SDAP as an intermediate observed upon the introduction of DDH were independent phenomena with each other, the change in color of broth resulted from dapD, and the disappearance of SDAP resulted from dapE. The relation between dapE and SDAP may be postulated judging from the biosynthesis pathway of L-lysine. The enhancement of dapF had no effect on the improvement in L-lysine productivity.

dapE was excised from pdapE, and it was inserted into pdapD to prepare a plasmid pMWdapDE1 containing both dapE and dapD (FIG. 23). Further, a fragment containing dapE and dapD was excised from pMWdapDE1, and it was inserted into pCAB1 to prepare pCABDE1 (FIG. 24). Strains harboring pCAB1, pCABDE1 or pCABD2 and a strain harboring both pCABDE1 and pdapF were prepared, and cultivation for L-lysine production was performed by using these strains. Results are shown in FIG. 17.

TABLE 17

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)	Color of broth	Accumulation of SDAP
W3110(tyrA)/pCAB1	10.80	26.5	brown	+
W3110(tyrA)/pCABDE1	12.23	30.0	yellow	−
W3110(tyrA)/pCABDE1 + pdapF	11.82	29.0	yellow	−
W3110(tyrA)/pCABD2	12.23	30.0	yellow	−

It was found that the L-lysine production amount, the color of broth, and the presence or absence of accumulation of SDAP became equivalent to those in the case of the production of DDH by enhancing dapD and dapE in combination. In addition, it was found that further enhancement of dapF had no effect on the improvement in L-lysine productivity, and the reaction participated by dapF did not make rate limitation. The results described above can be interpreted as follows.

Upon the step of introduction of pCAB1, intermediates are accumulated at two steps of SKAP (N-succinyl-ε-keto-L-α-aminopimelic acid) and SDAP. Among these intermediates, SDAP was detected in an extracellular broth. Although SKAP was not detected, it was speculated to be accumulated in bacterial cells. The reason for such speculation resides in the color of broth. The color of broth is yellow in the case of the wild type strain (W3110(tyrA)) or the like producing no L-lysine. However, the broth becomes brown probably due to bacteriolysis or the like when a load is applied to growth. It is speculated that SDAP has a small load on growth because it is discharged to the outside of cells, and hence, the broth is improved to have a yellow color although the accumulation amount of SDAP increases when SKAP is metabolized by the enhancement of only dapD. However, even if dapD is enhanced, the accumulation amount of L-lysine does not increase unless rate limitation by further downstream dapE is desensitized.

<6> Conclusion

According to the results described above, it has been found that the L-lysine productivity is improved in a stepwise manner by performing (1) introduction of dapA*, (2) introduction of lysC*, (3) enhancement of dapB, and (4) enhancement of DDH or dapD and dapE in bacteria belonging to the genus Escherichia. Further, *E. coli*, in which the L-lysine productivity is improved in a stepwise manner, has been obtained.

<7> Analysis of Rate Determining Step of L-lysine Biosynthesis System in *E. coli* C Strain

In order to examine whether or not the conclusion obtained in the foregoing could be applied to strains other than the *E. coli* K-12 series, rate determining steps of an L-lysine biosynthesis system of an *E. coli* C strain (IFO 13891) were analyzed in the same manner as described above. The cultivation condition was the same as that of W3110 (tyrA), however, L-tyrosine was not added to the medium.

(1) Identification of the First Rate Determining Step

The *E. coli* C strain (IFO 13891) transformed with plasmids containing genes of the L-lysine biosynthesis system was cultivated in the medium for L-lysine production, and the production amount of L-lysine hydrochloride was measured. Results are shown in Table 18.

TABLE 18

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yields versus sugar (%)
C	0 08	0 2
C/pppc	0 08	0 2
C/paspC	0 12	0 3
C/plysC	0 08	0 2
C/plysC*	0 12	0 3
C/pasd	0 08	0.2
C/pdapA	0 32	0.8
C/pdapA*	0 71	1 75
C/pdapB	0 12	0.3
C/pDDH	0.08	0.2
C/plysA	0.08	0.2

In the same manner as in W3110 (tyrA), L-lysine was also accumulated in the medium by the C strain by introducing the wild type dapA and further the inhibition-desensitized type dapA* lysC* had no effect on the L-lysine productivity.

(2) Identification of the Second Rate Determining Step p The plasmid RSF24P containing dapA* was introduced into the *E. coli* C strain, and plasmids containing genes of the L-lysine biosynthesis system were further introduced Obtained transformants were cultivated in the medium for L-lysine production, and the production amount of L-lysine hydrochloride was measured Results are shown in Table 19

TABLE 19

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
C/RSF24P	0.71	1 75
C/RSF24P + pppc	0.71	1 74
C/RSF24P + paspC	0.69	1 70
C/RSF24P + plysC	0.65	1 60
C/RSF24P + plysC*	1 82	4.50
C/RSF24P + pasd	0.70	1 73
C/RSF24P + pdapA	0.71	1 75
C/RSF24P + pdapA*	0.69	1 70
C/RSF24P + pdapB	0.99	2 45
C/RSF24P + pDDH	0.73	1 80
C/RSF24P + plysA	0.69	1 70

It was found that lysC* had an effect on the improvement in L-lysine productivity even in the case of the C strain with transformed dapA*, and the reaction participated by lysC* was the second rate determining step.

(3) Identification of the Third Rate Determining Step

The plasmid RSFD80 containing dapA* and lysC* was introduced into the *E. coli* C strain, and plasmids containing genes of the L-lysine biosynthesis system were further introduced. Obtained transformants were cultivated in the medium for L-lysine production, and the production amount of L-lysine hydrochloride was measured Results are shown in Table 20

TABLE 20

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
C/RSFD80	1 82	4 5
C/RSFD80 + pppc	1 74	4 3
C/RSFD80 + paspC	1 82	4 5
C/RSFD80 + plysC	1 91	4 7

TABLE 20-continued

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
C/RSFD80 + plysC*	1.74	4 3
C/RSFD80 + pasd	1.82	4 5
C/RSFD80 + pdapA	1.95	4 8
C/RSFD80 + pdapA*	1.91	4.7
C/RSFD80 + pdapB	2.31	5.7
C/RSFD80 + pDDH	2.15	5.3
C/RSFD80 + plysA	1 95	4.8

In the same manner as in the W3110 strain, only dapB had an effect on the improvement in L-lysine productivity, and it was found to be the third rate determining step.

(4) Identification of the Fourth Rate Determining Step

The plasmid pCAB1 containing dapA*, lysC* and dapB was introduced into the *E. coli* C strain, and plasmids containing genes of the L-lysine biosynthesis system were further introduced. Obtained transformants were cultivated in the L-lysine-producing medium, and the production amount of L-lysine hydrochloride was measured. Results are shown in Table 21

TABLE 21

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
C/pCAB1	2.31	5 7
C/pCAB1 + pppc	2.23	5 5
C/pCAB1 + paspC	2.35	5 8
C/pCAB1 + plysC	2.27	5 6
C/pCAB1 + plysC*	2.19	5 4
C/pCAB1 + pasd	2.23	5 5
C/pCAB1 + pdapA	2.31	5 7
C/pCAB1 + pdapA*	2.27	5 6
C/pCAB1 + pdapB	2.23	5 5
C/pCAB1 + pDDH	2.59	6 4
C/pCAB1 + plysA	2.19	5 4

In the same manner as in the W3110 strain, only DDH had an effect on the improvement in L-lysine productivity, and it was found to be the fourth rate determining step.

(5) Analysis of Rate Determining Steps Among dapC, dapD, dapE and dapF

Plasmid harboring the dapD, dapE or dapF genes were introduced, instead of DDH, into the *E. coli* C strain harboring pCAB1, and cultivation for L-lysine production was performed Results are shown in Table 22.

TABLE 22

Bacterial strain	Production amount of L-lysine hydro-chloride (g/l)	Yield versus sugar (%)	Color of broth	Accumulation of SDAP
C/pCAB1	2.31	5 7	brown	+
C/pCAB1 + pdapD	2.43	6.0	yellow	+
C/pCAB1 + pdapE	2.35	5 8	brown	–
C/pCAB1 + pdapF	2.23	5 5	brown	+
C/pCABDE1	2.59	6 4	yellow	–
C/pCABDE1 + pdapF	2.43	6.0	yellow	–
C/pCABD2	2.59	6.4	yellow	–

It was found that the two steps of dapD and dapE also concerned the rate determining in the C strain in the same manner as in the W3110 strain.

As described above, the strains of K-12 and C belonging to the different series had the same rate determining order.

Thus it is believed that the entire species of *E. coli* can be applied with the concept that the L-lysine productivity can be improved in a stepwise manner by performing introduction of dapA* and lysC* and enhancement of dapB and DDH (or dapD and dapE) in this order

Industrial Applicability

According to the present invention, there has been obtained a DDPS mutant gene originating from a bacterium belonging to the genus Escherichia in which feedback inhibition by L-lysine is sufficiently desensitized. An L-lysine-producing bacterium more improved than those in the prior art has been able to be obtained by introducing the gene into a bacterium belonging to the genus Escherichia harboring an aspartokinase in which feedback inhibition by L-lysine is desensitized.

Further, the L-lysine productivity can be improved in a stepwise manner by enhancing dapB and DDH (or dapD and dapE) of the aforementioned L-lysine-producing bacterium in this order.

SEQUENCE LISTING

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(iii) NUMBER OF SEQUENCES: 20

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

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(2) INFORMATION FOR SEQ ID NO:2:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(2) INFORMATION FOR SEQ ID NO:3:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
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(B) STRAIN: MC1061

(ix) FEATURE:
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(B) LOCATION: 248
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(ix) FEATURE:

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(A) NAME/KEY: primer_bind
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(D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: E"

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(B) LOCATION: 1156..1175
(D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: E"

(ix) FEATURE:
(A) NAME/KEY: RBS
(B) LOCATION: 261..265
(D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: S"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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TGCATACAAC AATCAGAACG GTTCTGTCTG CTTGCTTTTA ATGCCATACC AAACGTACCA    240

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(2) INFORMATION FOR SEQ ID NO:4:

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(A) LENGTH: 292 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Ala Thr Leu Asn His Asp Glu His Ala Asp Val Val Met Met Thr Leu
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Ile Val Gly Cys Leu Thr Val Thr Pro Tyr Tyr Asn Arg Pro Ser Gln
            100                 105                 110

Glu Gly Leu Tyr Gln His Phe Lys Ala Ile Ala Glu His Thr Asp Leu
        115                 120                 125

Pro Gln Ile Leu Tyr Asn Val Pro Ser Arg Thr Gly Cys Asp Leu Leu
    130                 135                 140

Pro Glu Thr Val Gly Arg Leu Ala Lys Val Lys Asn Ile Ile Gly Ile
145                 150                 155                 160

Lys Glu Ala Thr Gly Asn Leu Thr Arg Val Asn Gln Ile Lys Glu Leu
                165                 170                 175

Val Ser Asp Asp Phe Val Leu Leu Ser Gly Asp Asp Ala Ser Ala Leu
            180                 185                 190

Asp Phe Met Gln Leu Gly Gly His Gly Val Ile Ser Val Thr Thr Asn
        195                 200                 205

Val Ala Ala Arg Asp Met Ala Gln Met Cys Lys Leu Ala Ala Glu Glu
    210                 215                 220

His Phe Ala Glu Ala Arg Val Ile Asn Gln Arg Leu Met Pro Leu His
225                 230                 235                 240
```

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```
Asn Lys Leu Phe Val Glu Pro Asn Pro Ile Pro Val Lys Trp Ala Cys
                245                 250                 255

Lys Glu Leu Gly Leu Val Ala Thr Asp Thr Leu Arg Leu Pro Met Thr
            260                 265                 270

Pro Ile Thr Asp Ser Gly Arg Glu Thr Val Arg Ala Ala Leu Lys His
        275                 280                 285

Ala Gly Leu Leu
    290
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
CTTCCCTTGT GCCAAGGCTG                                          20
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
GAATTCCTTT GCGAGCAG                                            18
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2147 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ESCHERICHIA COLI
(B) STRAIN: MC1061

(ix) FEATURE:
(A) NAME/KEY: -35_signal
(B) LOCATION: 242..249
(D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: S"

(ix) FEATURE:
(A) NAME/KEY: -10_signal
(B) LOCATION: 265..273
(D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: S"

(ix) FEATURE:
(A) NAME/KEY: primer_bind
(B) LOCATION: 536..555
(D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: E"

(ix) FEATURE:
(A) NAME/KEY: RBS
(B) LOCATION: 575..578
(D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: S"

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(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 584..1933
(D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: S"

(ix) FEATURE:
(A) NAME/KEY: terminator
(B) LOCATION: 1941..1968
(D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: S"

(ix) FEATURE:
(A) NAME/KEY: primer_bind
(B) LOCATION: 2128..2147
(D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: E"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
TCGAAGTGTT TCTGTAGTGC CTGCCAGGCA GCGGTCTGCG TTGGATTGAT GTTTTTCATT     60

AGCAATACTC TTCTGATTTT GAGAATTGTG ACTTTGGAAG ATTGTAGCGC CAGTCACAGA    120

AAAATGTGAT GGTTTTAGTG CCGTTAGCGT AATGTTGAGT GTAAACCCTT AGCGCAGTGA    180

AGCATTTATT AGCTGAACTA CTGACCGCCA GGAGTGGATG AAAAATCCGC ATGACCCCAT    240

CGTTGACAAC CGCCCCGCTC ACCCTTTATT TATAAATGTA CTACCTGCGC TAGCGCAGGC    300

CAGAAGAGGC GCGTTGCCCA AGTAACGGTG TTGGAGGAGC CAGTCCTGTG ATAACACCTG    360

AGGGGGTGCA TCGCCGAGGT GATTGAACGG CTGGCCACGT TCATCATCGG CTAAGGGGGC    420

TGAATCCCCT GGGTTGTCAC CAGAAGCGTT CGCAGTCGGG CGTTTCGCAA GTGGTGGAGC    480

ACTTCTGGGT GAAAATAGTA GCGAAGTATC GCTCTGCGCC CACCCGTCTT CCGCTCTTCC    540

CTTGTGCCAA GGCTGAAAAT GGATCCCCTG ACACGAGGTA GTT ATG TCT GAA ATT      595
                                                Met Ser Glu Ile
                                                  1

GTT GTC TCC AAA TTT GGC GGT ACC AGC GTA GCT GAT TTT GAC GCC ATG      643
Val Val Ser Lys Phe Gly Gly Thr Ser Val Ala Asp Phe Asp Ala Met
  5                  10                  15                  20

AAC CGC AGC GCT GAT ATT GTG CTT TCT GAT GCC AAC GTG CGT TTA GTT      691
Asn Arg Ser Ala Asp Ile Val Leu Ser Asp Ala Asn Val Arg Leu Val
                 25                  30                  35

GTC CTC TCG GCT TCT GCT GGT ATC ACT AAT CTG CTG GTC GCT TTA GCT      739
Val Leu Ser Ala Ser Ala Gly Ile Thr Asn Leu Leu Val Ala Leu Ala
             40                  45                  50

GAA GGA CTG GAA CCT GGC GAG CGA TTC GAA AAA CTC GAC GCT ATC CGC      787
Glu Gly Leu Glu Pro Gly Glu Arg Phe Glu Lys Leu Asp Ala Ile Arg
         55                  60                  65

AAC ATC CAG TTT GCC ATT CTG GAA CGT CTG CGT TAC CCG AAC GTT ATC      835
Asn Ile Gln Phe Ala Ile Leu Glu Arg Leu Arg Tyr Pro Asn Val Ile
     70                  75                  80

CGT GAA GAG ATT GAA CGT CTG CTG GAG AAC ATT ACT GTT CTG GCA GAA      883
Arg Glu Glu Ile Glu Arg Leu Leu Glu Asn Ile Thr Val Leu Ala Glu
 85                  90                  95                 100

GCG GCG GCG CTG GCA ACG TCT CCG GCG CTG ACA GAT GAG CTG GTC AGC      931
Ala Ala Ala Leu Ala Thr Ser Pro Ala Leu Thr Asp Glu Leu Val Ser
                105                 110                 115

CAC GGC GAG CTG ATG TCG ACC CTG CTG TTT GTT GAG ATC CTG CGC GAA      979
His Gly Glu Leu Met Ser Thr Leu Leu Phe Val Glu Ile Leu Arg Glu
            120                 125                 130

CGC GAT GTT CAG GCA CAG TGG TTT GAT GTA CGT AAA GTG ATG CGT ACC     1027
Arg Asp Val Gln Ala Gln Trp Phe Asp Val Arg Lys Val Met Arg Thr
        135                 140                 145

AAC GAC CGA TTT GGT CGT GCA GAG CCA GAT ATA GCC GCG CTG GCG GAA     1075
Asn Asp Arg Phe Gly Arg Ala Glu Pro Asp Ile Ala Ala Leu Ala Glu
    150                 155                 160
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CTG GCC GCG CTG CAG CTG CTC CCA CGT CTC AAT GAA GGC TTA GTG ATC     1123
Leu Ala Ala Leu Gln Leu Leu Pro Arg Leu Asn Glu Gly Leu Val Ile
165                 170                 175                 180

ACC CAG GGA TTT ATC GGT AGC GAA AAT AAA GGT CGT ACA ACG ACG CTT     1171
Thr Gln Gly Phe Ile Gly Ser Glu Asn Lys Gly Arg Thr Thr Thr Leu
                185                 190                 195

GGC CGT GGA GGC AGC GAT TAT ACG GCA GCC TTG CTG GCG GAG GCT TTA     1219
Gly Arg Gly Gly Ser Asp Tyr Thr Ala Ala Leu Leu Ala Glu Ala Leu
            200                 205                 210

CAC GCA TCT CGT GTT GAT ATC TGG ACC GAC GTC CCG GGC ATC TAC ACC     1267
His Ala Ser Arg Val Asp Ile Trp Thr Asp Val Pro Gly Ile Tyr Thr
        215                 220                 225

ACC GAT CCA CGC GTA GTT TCC GCA GCA AAA CGC ATT GAT GAA ATC GCG     1315
Thr Asp Pro Arg Val Val Ser Ala Ala Lys Arg Ile Asp Glu Ile Ala
    230                 235                 240

TTT GCC GAA GCG GCA GAG ATG GCA ACT TTT GGT GCA AAA GTA CTG CAT     1363
Phe Ala Glu Ala Ala Glu Met Ala Thr Phe Gly Ala Lys Val Leu His
245                 250                 255                 260

CCG GCA ACG TTG CTA CCC GCA GTA CGC AGC GAT ATC CCG GTC TTT GTC     1411
Pro Ala Thr Leu Leu Pro Ala Val Arg Ser Asp Ile Pro Val Phe Val
                265                 270                 275

GGC TCC AGC AAA GAC CCA CGC GCA GGT GGT ACG CTG GTG TGC AAT AAA     1459
Gly Ser Ser Lys Asp Pro Arg Ala Gly Gly Thr Leu Val Cys Asn Lys
            280                 285                 290

ACT GAA AAT CCG CCG CTG TTC CGC GCT CTG GCG CTT CGT CGC AAT CAG     1507
Thr Glu Asn Pro Pro Leu Phe Arg Ala Leu Ala Leu Arg Arg Asn Gln
        295                 300                 305

ACT CTG CTC ACT TTG CAC AGC CTG AAT ATG CTG CAT TCT CGC GGT TTC     1555
Thr Leu Leu Thr Leu His Ser Leu Asn Met Leu His Ser Arg Gly Phe
    310                 315                 320

CTC GCG GAA GTT TTC GGC ATC CTC GCG CGG CAT AAT ATT TCG GTA GAC     1603
Leu Ala Glu Val Phe Gly Ile Leu Ala Arg His Asn Ile Ser Val Asp
325                 330                 335                 340

TTA ATC ACC ACG TCA GAA GTG AGC GTG GCA TTA ACC CTT GAT ACC ACC     1651
Leu Ile Thr Thr Ser Glu Val Ser Val Ala Leu Thr Leu Asp Thr Thr
                345                 350                 355

GGT TCA ACC TCC ACT GGC GAT ACG TTG CTG ACG CAA TCT CTG CTG ATG     1699
Gly Ser Thr Ser Thr Gly Asp Thr Leu Leu Thr Gln Ser Leu Leu Met
            360                 365                 370

GAG CTT TCC GCA CTG TGT CGG GTG GAG GTG GAA GAA GGT CTG GCG CTG     1747
Glu Leu Ser Ala Leu Cys Arg Val Glu Val Glu Glu Gly Leu Ala Leu
        375                 380                 385

GTC GCG TTG ATT GGC AAT GAC CTG TCA AAA GCC TGC GGC GTT GGC AAA     1795
Val Ala Leu Ile Gly Asn Asp Leu Ser Lys Ala Cys Gly Val Gly Lys
    390                 395                 400

GAG GTA TTC GGC GTA CTG GAA CCG TTC AAC ATT CGC ATG ATT TGT TAT     1843
Glu Val Phe Gly Val Leu Glu Pro Phe Asn Ile Arg Met Ile Cys Tyr
405                 410                 415                 420

GGC GCA TCC AGC CAT AAC CTG TGC TTC CTG GTG CCC GGC GAA GAT GCC     1891
Gly Ala Ser Ser His Asn Leu Cys Phe Leu Val Pro Gly Glu Asp Ala
                425                 430                 435

GAG CAG GTG GTG CAA AAA CTG CAT AGT AAT TTG TTT GAG TAA             1933
Glu Gln Val Val Gln Lys Leu His Ser Asn Leu Phe Glu  *
            440                 445                 450

ATACTGTATG GCCTGGAAGC TATATTTCGG GCCGTATTGA TTTTCTTGTC ACTATGCTCA   1993

TCAATAAACG AGCCTGTACT CTGTTAACCA GCGTCTTTAT CGGAGAATAA TTGCCTTTAA   2053

TTTTTTTATC TGCATCTCTA ATTAATTATC GAAAGAGATA AATAGTTAAG AGAAGGCAAA   2113
```

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ATGAATATTA TCAGTTCTGC TCGCAAAGGA ATTC 2147

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 449 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Ser Glu Ile Val Val Ser Lys Phe Gly Gly Thr Ser Val Ala Asp
  1               5                  10                  15

Phe Asp Ala Met Asn Arg Ser Ala Asp Ile Val Leu Ser Asp Ala Asn
             20                  25                  30

Val Arg Leu Val Val Leu Ser Ala Ser Ala Gly Ile Thr Asn Leu Leu
         35                  40                  45

Val Ala Leu Ala Glu Gly Leu Glu Pro Gly Glu Arg Phe Glu Lys Leu
     50                  55                  60

Asp Ala Ile Arg Asn Ile Gln Phe Ala Ile Leu Glu Arg Leu Arg Tyr
 65                  70                  75                  80

Pro Asn Val Ile Arg Glu Glu Ile Glu Arg Leu Leu Glu Asn Ile Thr
                 85                  90                  95

Val Leu Ala Glu Ala Ala Ala Leu Ala Thr Ser Pro Ala Leu Thr Asp
            100                 105                 110

Glu Leu Val Ser His Gly Glu Leu Met Ser Thr Leu Leu Phe Val Glu
        115                 120                 125

Ile Leu Arg Glu Arg Asp Val Gln Ala Gln Trp Phe Asp Val Arg Lys
    130                 135                 140

Val Met Arg Thr Asn Asp Arg Phe Gly Arg Ala Glu Pro Asp Ile Ala
145                 150                 155                 160

Ala Leu Ala Glu Leu Ala Ala Leu Gln Leu Leu Pro Arg Leu Asn Glu
                165                 170                 175

Gly Leu Val Ile Thr Gln Gly Phe Ile Gly Ser Glu Asn Lys Gly Arg
            180                 185                 190

Thr Thr Thr Leu Gly Arg Gly Gly Ser Asp Tyr Thr Ala Ala Leu Leu
        195                 200                 205

Ala Glu Ala Leu His Ala Ser Arg Val Asp Ile Trp Thr Asp Val Pro
    210                 215                 220

Gly Ile Tyr Thr Thr Asp Pro Arg Val Val Ser Ala Ala Lys Arg Ile
225                 230                 235                 240

Asp Glu Ile Ala Phe Ala Glu Ala Ala Glu Met Ala Thr Phe Gly Ala
                245                 250                 255

Lys Val Leu His Pro Ala Thr Leu Leu Pro Ala Val Arg Ser Asp Ile
            260                 265                 270

Pro Val Phe Val Gly Ser Ser Lys Asp Pro Arg Ala Gly Gly Thr Leu
        275                 280                 285

Val Cys Asn Lys Thr Glu Asn Pro Pro Leu Phe Arg Ala Leu Ala Leu
    290                 295                 300

Arg Arg Asn Gln Thr Leu Leu Thr Leu His Ser Leu Asn Met Leu His
305                 310                 315                 320

Ser Arg Gly Phe Leu Ala Glu Val Phe Gly Ile Leu Ala Arg His Asn
                325                 330                 335

Ile Ser Val Asp Leu Ile Thr Thr Ser Glu Val Ser Val Ala Leu Thr
            340                 345                 350
```

-continued

Leu Asp Thr Thr Gly Ser Thr Ser Thr Gly Asp Thr Leu Leu Thr Gln
355 360 365

Ser Leu Leu Met Glu Leu Ser Ala Leu Cys Arg Val Glu Val Glu Glu
370 375 380

Gly Leu Ala Leu Val Ala Leu Ile Gly Asn Asp Leu Ser Lys Ala Cys
385 390 395 400

Gly Val Gly Lys Glu Val Phe Gly Val Leu Glu Pro Phe Asn Ile Arg
405 410 415

Met Ile Cys Tyr Gly Ala Ser Ser His Asn Leu Cys Phe Leu Val Pro
420 425 430

Gly Glu Asp Ala Glu Gln Val Val Gln Lys Leu His Ser Asn Leu Phe
435 440 445

Glu
450

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTTTCACTGA TATCCCTCCC 20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAAAAGTGGA CCAAATGGTC 20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CATCTAAGTA TGCATCTCGG 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGCCCCTCGA GCTAAATTAG 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGCACGGTAG GATGTAATCG 20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTAATGAAAC AAATGCCCGG 20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTTATTCATA ATTGCCACCG 20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CACGGTAATA CATATAACCG 20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCTGCAATTG TCAAACGTCC 20

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTCGACGCGC TTGAGATCTT 20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCATAAAGAG TCGCTAAACG 20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAACCGCCCG GTCATCAAGC 20

What is claimed is:

1. An isolated DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus Escherichia, wherein the dihydrodipicolinate synthase has a mutation which desensitizes feedback inhibition by L-lysine, wherein the mutation is selected from the group consisting of

(a) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,

(b) a mutation to replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue, and

(c) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue and replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,

(d) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,

(e) a mutation to replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue, and

(f) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate

synthase of SEQ ID NO: 4 with another amino acid residue and replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue

2. The isolated DNA of claim 1, wherein the mutation to desensitize feedback inhibition by L-lysine is selected from the group consisting of

(a) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue,

(b) a mutation to replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue, and

(c) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue and replace the 118th histidine residue as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue,

(d) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue,

(e) a mutation to replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue, and

(f) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue and replace the histidine residue corresponding to the 118th residue as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue

3. A bacterium belonging the genus Escherichia which is transformed with a DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus Escherichia and having mutation to desensitize feedback inhibition by L-lysine, wherein the mutation is selected from the group consisting of

(a) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,

(b) a mutation to replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue, and

(c) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue and replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,

(d) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,

(e) a mutation to replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue, and

(f) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue and replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue

4. The bacterium of claim 3, wherein the mutation is selected from the group consisting of

(a) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue,

(b) a mutation to replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue, and

(c) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue and replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue,

(d) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue,

(e) a mutation to replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue, and

(f) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue and replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue

5. The bacterium of claim 3, further harboring an aspartokinase which is desensitized to feedback inhibition by L-lysine.

6. The bacterium of claim 5, which is obtained by introducing, into its cells, a DNA coding for an aspartokinase III originating from a bacterium belonging to the genus Escherichia, wherein the aspartokinase III has a mutation which desensitizes feedback inhibition by L-lysine.

7. The bacterium of claim 6, wherein the mutation is selected from the group consisting of

(a) a mutation to replace the glycine residue at the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(b) a mutation to replace the glycine residue at the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO:8 with another amino acid residue and replace the glycine residue at the 408th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(c) a mutation to replace the glycine residue at the 34th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue and replace the glycine residue at the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(d) a mutation to replace the leucine residue at the 325th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(e) a mutation to replace the methionine residue at the 318th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(f) a mutation to replace the methionine residue at the 318th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue and replace the valine residue at the 349th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(g) a mutation to replace the serine residue at the 345th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(h) a mutation to replace the valine residue at the 347th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(i) a mutation to replace the threonine residue at the 352nd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(j) a mutation to replace the threonine residue at the 352nd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue and replace the serine residue at the 369th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(k) a mutation to replace the glutamic acid residue at the 164th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue, and

(l) a mutation to replace the methionine residue at the 417th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue and replace the cysteine residue at the 419th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(m) a mutation to replace the glycine residue corresponding to the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(n) a mutation to replace the glycine residue corresponding to the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO:8 with another amino acid residue and replace the glycine residue corresponding to the 408th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(o) a mutation to replace the glycine residue corresponding to the 34th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO:8 with another amino acid residue and replace the glycine residue corresponding to the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(p) a mutation to replace the leucine residue corresponding to the 325th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(q) a mutation to replace the methionine residue corresponding to the 318th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(r) a mutation to replace the methionine residue corresponding to the 318th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue and replace the valine residue corresponding to the 349th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(s) a mutation to replace the serine residue corresponding to the 345th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(t) a mutation to replace the valine residue corresponding to the 347th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(u) a mutation to replace the threonine residue corresponding to the 352nd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(v) a mutation to replace the threonine residue corresponding to the 352nd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue and replace the serine residue corresponding to the 369th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

(w) a mutation to replace the glutamic acid residue corresponding to the 164th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue, and

(x) a mutation to replace the methionine residue corresponding to the 417th position as counted from the

N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue and replace the cysteine residue corresponding to the 419th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue.

8. The bacterium of claim 7, wherein the mutation is selected from the group consisting of

(a) a mutation to replace the glycine residue at the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an aspartic acid residue,

(b) a mutation to replace the glycine residue at the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an aspartic acid residue and replace the glycine residue at the 408th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an aspartic acid residue,

(c) a mutation to replace the arginine residue at the 34th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO:8 with a cysteine residue and replace the glycine residue at the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an aspartic acid residue,

(d) a mutation to replace the leucine residue at the 325th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a phenylalanine residue,

(e) a mutation to replace the methionine residue at the 318th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an isoleucine residue,

(f) a mutation to replace the methionine residue at the 318th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an isoleucine residue and replace the valine residue at the 349th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a methionine residue,

(g) a mutation to replace the serine residue at the 345th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a leucine residue,

(h) a mutation to replace the valine residue at the 347th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a valine residue,

(i) a mutation to replace the threonine residue at the 352nd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an isoleucine residue,

(j) a mutation to replace the threonine residue at the 352nd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an isoleucine residue and replace the serine residue at the 369th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a phenylalanine residue,

(k) a mutation to replace the glutamic acid residue at the 164th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a lysine residue, and

(l) a mutation to replace the methionine residue at the 417th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an isoleucine residue and replace the cysteine residue at the 419th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a tyrosine residue,

(m) a mutation to replace the glycine residue corresponding to the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an aspartic acid residue,

(n) a mutation to replace the glycine residue corresponding to the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an aspartic acid residue and replace the glycine residue corresponding to the 408th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an aspartic acid residue,

(o) a mutation to replace the arginine residue corresponding to the 34th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO:8 with a cysteine residue and replace the glycine residue corresponding to the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an aspartic acid residue,

(p) a mutation to replace the leucine residue corresponding to the 325th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a phenylalanine residue,

(q) a mutation to replace the methionine residue corresponding to the 318th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an isoleucine residue,

(r) a mutation to replace the methionine residue corresponding to the 318th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an isoleucine residue and replace the valine residue corresponding to the 349th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a methionine residue,

(s) a mutation to replace the serine residue corresponding to the 345th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a leucine residue,

(t) a mutation to replace the valine residue corresponding to the 347th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a valine residue,

(u) a mutation to replace the threonine residue corresponding to the 352nd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an isoleucine residue,

(v) a mutation to replace the threonine residue corresponding to the 352nd position as counted from the N-terminal in the amino acid sequence of the aspar-

tokinase III of SEQ ID NO: 8 with an isoleucine residue and replace the serine residue corresponding to the 369th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a phenylalanine residue,

(w) a mutation to replace the glutamic acid residue corresponding to the 164th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a lysine residue, and

(x) a mutation to replace the methionine residue corresponding to the 417th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an isoleucine residue and replace the cysteine residue corresponding to the 419th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a tyrosine residue.

9. The bacterium of claim **5**, wherein a dihydrodipicolinate reductase gene is enhanced

10. The bacterium of claim **9**, transformed with a recombinant DNA constructed by ligating the dihydrodipicolinate reductase gene with a vector autonomously replicable in cells of bacteria belonging to the genus Escherichia.

11. The bacterium of claim **9**, into which an enhanced diaminopimelate dehydrogenase gene originating from coryneform bacterium has been introduced

12. The of claim **11**, transformed with a recombinant DNA constructed by ligating the diaminopimelate dehydrogenase gene originating from a coryneform bacterium with a vector autonomously replicable in cells of bacteria belonging to the genus Escherichia.

13. The bacterium of claim **9**, wherein a succinyldiaminopimelate transaminase gene and a succinyldiaminopimelate transaminase gene and a succinyldiaminopimelate deacylase gene are enhanced.

14. The bacterium of claim **13**, transformed with a single recombinant DNA or two recombinant DNA's constructed by ligating the succinyldiaminopimelate transaminase gene and the succinyldiaminopimelate deacylase gene with an identical vector or different vectors autonomously replicable in cells of bacteria belonging to the genus Escherichia.

15. A method of producing L-lysine, comprising:

cultivating the bacterium of claim **3** in a suitable culture medium, producing and accumulating L-lysine in the culture thereof, and collecting L-lysine from the culture.

16. A bacterium belonging the genus Escherichia which is transformed with a DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus Escherichia and having mutation to desensitize feedback inhibition by L-lysine, and

further harboring an aspartokinase which is desensitized to feedback inhibition by L-lysine, and

wherein a dihydrodipicolinate reductase gene is enhanced.

17. The bacterium of claim **16**, transformed with a recombinant DNA constructed by ligating the dihydrodipicolinate reductase gene with a vector autonomously replicable in cells of bacteria belonging to the genus Escherichia.

18. The bacterium of claim **16**, into which an enhanced diaminopimelate dehydrogenase gene originating from coryneform bacterium has been introduced.

19. The of claim **18**, transformed with a recombinant DNA constructed by ligating the diaminopimelate dehydrogenase gene originating from a coryneform bacterium with a vector autonomously replicable in cells of bacteria belonging to the genus Escherichia.

20. The bacterium of claim **16**, wherein a succinyldiaminopimelate transaminase gene and a succinyldiaminopimelate transaminase gene and a succinyldiaminopimelate deacylase gene are enhanced.

21. The bacterium of claim **20**, transformed with a single recombinant DNA or two recombinant DNA's constructed by ligating the succinyldiaminopimelate transaminase gene and the succinyldiaminopimelate deacylase gene with an identical vector or different vectors autonomously replicable in cells of bacteria belonging to the genus Escherichia

22. A method of producing L-lysine, comprising:

cultivating the bacterium of claim **16** in a suitable culture medium, producing and accumulating L-lysine in the culture thereof, and collecting L-lysine from the culture.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,040,160
DATED : March 21, 2000
INVENTOR(S) : Kojima et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 67,
Line 34-35, "succinyldiaminopimelate transaminase" should read
-- tetrahydrodipicolinate succinylase --.
Lines 35-36, delete "and a succinyldiaminopimelate transaminase gene".
Line 40, "succinyldiaminopimelate transaminase" should read
-- tetrahydrodipicolinate succinylase --.

Column 68,
Line 3, "producing and accumulating" should read -- to produce and accumulate --.
Lines 26-27, "succinyldiaminopimelate transaminase" should read
-- tetrahydrodipicolinate succinylase --.
Lines 27-28, delete "and a succinyldiaminopimelate transaminase gene".
Line 32, "succinyldiaminopimelate transaminase" should read
-- tetrahydrodipicolinate succinylase --.
Line 38, "producing and accumulating" should read -- to produce and accumulate --.

Signed and Sealed this

Second Day of July, 2002

Attest:

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office